TWO-STAGE ACIDIC-ALKALINE PRETREATMENT OF *MISCANTHUS* FOR BIOETHANOL PRODUCTION

BY

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DISSERTATION

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Abstract

Pretreatment is the rate-limiting step for bioethanol production from lignocellulosic biomass, and subsequently intensive studies have been undertaken to improve the pretreatment efficiency. However, so far most pretreatment methods failed to achieve desirable sugar recovery from both cellulose and hemicellulose in the biomass, which is essential to improve process economics and competitiveness of bioethanol. To address the issue, this research developed two innovative pretreatment methods successively. *Miscanthus* was used as the model feedstock. The effects of primary pretreatment conditions on the performance were examined. Process optimization was conducted to locate the best operational conditions. The pretreatment effectiveness was evaluated in terms of sugars yield, biomass structure alteration and ethanol yield.

A two-stage acidic-alkaline pretreatment was proposed to obtain most intact monosaccharides from cellulose and hemicellulose. Dilute sulfuric acid pretreatment was performed in the first stage mainly for hemicellulose removal while the second stage carried out lime pretreatment primarily for delignification. The process was optimized by using Response Surface Methodology (RSM) analysis taking account of temperature, catalyst loading and residence time. It was demonstrated that the maximized sugars yield could be attained at medium severities in acid stage and higher severities in alkaline stage. The best pretreatment conditions were found at 0.73 wt% H_2SO_4, 150 ºC, 6 min in acid stage, and 0.024 g/g dry biomass of lime loading, 202 ºC in alkaline stage. In addition to the greatly improved sugars yield, the two-stage process also showed great promises in considerably reduced induction of primary degradation by-products, with proven significantly enhanced ethanol yield.

To further improve hemicellulose hydrolysis in acid stage, a second pretreatment method, combined acid hydrolysis, was developed to replace the conventional dilute acid pretreatment. The applied combined acid catalysts included sulfuric acid and two biomimetic acids, trifluoroacetic acid (TFA) and maleic acid (MA), respectively. The influences of acid blending ratio, temperature, and acid dosage on pretreatment performance were investigated. Synergistic effects on hemicellulose decomposition were observed under all studied conditions. Further, combined TFA pretreatment could

efficiently prevent xylose degradation. Combined acid hydrolysis was shown to be a favorable pretreatment method for its improved xylose yield, reduced catalyst costs and enhanced ethanol yield. Ultimately, further study indicated adoption of combined acid hydrolysis in the two-stage acidic-alkaline pretreatment could achieve higher sugars recovery.

To Mum and Wenting, the two
most important ladies in my life

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CHAPTER 1
INTRODUCTION

Relying on conventional fossil fuels for the entire energy supply were problematic to the United States and worldwide, due to the severe consequences they may cause, such as potential energy shortage and jeopardizing national energy security. In this regard, exploitation and development of alternative energy resources are highly demanded and fully underway to supplement the fossil fuels and diverse the energy portfolio. Biofuels - liquid fuels derived from biomass - so far are important alternative energy sources (Service, 2007), with bioethanol as the dominant type. Seen as "fuel of the future" by Henry Ford (Kovarik, 1998), ethanol is an important and most popular alternative fuel in the transportation sector for several benefits. Ethanol as well as incomplete oxidation by-products are not as toxic as gasoline (Minteer, 2006), and can also be easily incorporated into the existing infrastructure as a blend with gasoline in concentrations between 10-85%, or even as a pure fuel in dedicated engines (Galbe and Zacchi, 2007). Moreover, ethanol can be produced from a variety of biomass sources. According to the Renewable Fuels Association (RFA), annual ethanol fuel production in the U.S. saw a steadily exponential growth over the past three decades with an average 11% annual growth rate up to roughly 14 billion gallons in 2008 (Figure 1.1), representing about 10% of the U.S. gasoline supply. In view of the rapid growth of biofuels and with a great vision of their use as alternative fuels, the U.S. government set a more ambitious goal to increase biofuels production to 36 billion gallons by 2022 (The Energy Independence and Security Act, 2007) and replace 30% of U.S. gasoline with ethanol by 2030 (Energy Policy Act, 2005).

1.1 Feedstocks for Bioethanol Production

Although generated from a wide range of feedstock sources, nearly all bioethanols worldwide nowadays are first generation biofuels, which are made from sugar- or starch-based feedstocks, mainly food crops. In the United States, currently more than 90% of the ethanol comes from corn (U.S. Department of Energy, Alternative Fuels & Advanced Vehicles Data Center). Ethanol production from food crops such as corn has been

considered not sustainable due to intensive land use and the potential consequences of food shortages and price rise. In addition, food crops are not so environmental friendly as previously claimed since they need vast amount of water to grow and may only lead to marginal greenhouse gas mitigation (Galbe and Zacchi, 2007).

As promising alternatives of unsustainable corn ethanol, second generation biofuels has been advocated as the substitutes. They are made from non-food crops, mainly from lignocellulosic biomass. An abundant global biomass source, up to date lignocellulose is largely unutilized and considered generating low level of net greenhouse gas emissions (Galbe and Zacchi, 2007). The global production of lignocellulose (3-5 Gt/yr) could potentially provide 10-20% of current world energy demand (Lange, 2007). Lignocellulosic biomass can be sorted into four main sources in a descending order of available amount: forest products and residues, agricultural residues, municipal paper waste, and dedicated energy crops. Although accounting for a small share of biomass at present, dedicated energy crops seem to be the largest biomass resource in the long term (Lin and Tanaka, 2006), due to the sizeable reduction of growing and harvesting energy crops from a single planting (Monique et al., 2003).

As a potential dedicated energy crop, *Miscanthus* has drew great attention and been intensively investigated in Europe since 1980s (Price et al., 2004; Fischer et al., 2005; Tuck et al., 2006; Lewandowski et al., 2000) and more recently in the U.S. It is a tall perennial rhizomatous grass with C4 photosynthetic pathway and subsequently has many favorable characteristics. Numerous studies throughout Europe showed that *Miscanthus* has great biomass yields of 10-40 t/ha depending on where it is grown and when it is harvested (Lewandowski et al., 2003). *Miscanthus x giganteus* was found to be among the most productive genotypes so far (Clifton-Brown et al., 2001b). In general, *Miscanthus* was found to have at least the same biomass yield as switchgrass, the primarily studied energy crop in the U.S. Although *Miscanthus* yield in the U.S. was little known, a high yield of this biomass even in cooler area in the Europe showed potentially widespread application in the northern U.S. (Beale et al., 1996) and most part of Midwest. In addition, this robust plant can highly tolerate salt, acidity and drought, and can grow in poor-quality soil. It requires little fertilizer, herbicide and water, and it is non-invasive (Lewandowski et al., 2000; Watanabe et al., 2006; Murnen et al., 2007). Previously,

Miscanthus was mostly studied as a fuel for electricity generation (Clifton-Brown et al., 2004). Only recently investigations of biochemical conversion process to bioethanol have been reported (Murnen et al., 2007; Guo et al., 2008; Sørensen et al., 2008; Brosse et al., 2009; de Vrije et al., 2002). Furthermore, another credit added to bioethanol production by *Miscanthus* is its higher cellulose content (similar to hardwoods) than most crops, which could raise the theoretical ethanol yield (Murnen et al., 2007).

1.2 Convertion Process

Generally, the process of converting lignocellulose to bio-ethanol consists of four major steps: pretreatment of biomass to break down the main components, hydrolysis to depolymerize the broken components into monosaccharides, fermentation of hexose and pentose to ethanol, and final ethanol distillation. Pretreatment is among the most difficult steps due to its technical and economic challenges.

First, lignocellulose is composed of three polymeric compounds: cellulose, hemicellulose, and lignin. The sugars to produce ethanol through fermentation are hexose (mainly glucose) and pentose (mainly xylose) derived from cellulose and hemicellulose individually. However, cellulose and hemicellulose are initially tightly bounded to lignin by hydrogen bonds and some covalent bonds, which form the backbone of lignocellulose (Lin and Tanaka, 2006). Besides, roughly 50-90% of cellulose in biomass stays in crystalline form which is recalcitrant to be broken down by hydrolytic enzyme (Jacobsen and Wyman, 2000). For that matter, to make cellulose and hemicellulose accessible for enzyme hydrolysis, pretreatment process is required to liberate these carbohydrate polymers from lignin, and convert crystalline cellulose to amorphous cellulose, which is suitable for hydrolysis. Meanwhile, due to its instability under pretreatment conditions, hemicellulose could be partly hydrolyzed to pentose. Secondly, pretreatment is the most costly step throughout the conversion processes, making up to 40% of the total processing cost (Zhang et al., 2009; Eggeman and Elander, 2005). Thirdly, since pretreatment is the first step of the conversion process, it strongly affects energy demand and costs of the downstream steps (Wyman et al., 2005b).

In fact, lignocellulose will not just end in cellulose, hemicellulose and their hexose and pentose products during pretreatment. Furfural and 5-hydroxymethylfurfural (HMF)

are formed from the degradation of pentoses and hexoses, respectively. Sometimes furfural may further convert to formic acid, while HMF to formic acid and levulinic acid (Galbe and Zacchi, 2007). Other than that, acetic acid, present originally in the form of acetylated sugars in hemicellulose, is also released during pretreatment (Taherzadeh and Karimi, 2007). Furthermore, some pretreatment processes could cause the substantial formation of lignin degradation products like phenolic and aromatic compounds (Palmqvist and Hahn-Hägerdal, 2000). All of the above by-products could present certain levels of inhibitory effects on the fermentation microorganisms (Palmqvist and Hahn-Hägerdal, 2000; Klinke et al., 2004). These compounds counteract the benefit of sugars uptake for ethanol production, thereby impact the ethanol production rate or reduce the end ethanol yield. The primary involved chemical conversion pathways throughout the pretreatment process were summarized in Figure 1.2.

1.3 Single-Stage Pretreatment

Since the pretreatment process is the rate-limiting step and the most challenging task, over the past 40 years, a number of technologies have been developed for lignocelluloses pretreatment, and so far those methods were reviewed extensively and systematically (Galbe and Zacchi, 2007; Sun and Sun, 2002; Mosier et al., 2005; Jørgensen et al., 2007; Taherzadeh and Karimi, 2008; Yang and Wyman, 2008; da Costa Sousa et al., 2009; Kumar et al., 2009; Pienkos and Zhang, 2009). They can be roughly divided into four categories: physical (e.g. mechanical comminution, irradiation, electrical pretreatment), chemical, physicochemical, and biological pretreatments. The large power consumption of mechanical comminution, high cost of irradiation and insufficient research of electrical methods put physical pretreatment alone in an unpractical position. Although being considered environmental friendly and saving energy, biological pretreatment sustains a fairly low processing rate which is intolerable for industrial application. Pretreatments with addition of chemicals were proved to be effective, however, a mixture of physical and chemical methods are more favored as physicochemical methods. Based on pH value of the applied catalyst, physicochemical pretrreatments can be further grouped into acid-based pretreatments, neutral pretreatments, alkaline pretreatments, and solvent based

pretreatments (Galbe and Zacchi, 2007; Jørgensen et al., 2007; Pienkos and Zhang, 2009). Various pretreatment technologies with their pros and cons were listed in Table 1..1.

As can be seen from the table, a wide range of technologies could be applied in the physicochemical pretreatment. Each technology has its own benefits and limitations, but all of the methods share two common technical barriers:

(1) Preference to treat specific components.

It is clearly to see from Table 1..1, the preference to degrade one specific component could be classified based on the pretreatment categories. Acid-based pretreatment methods, such as SO_2-catalyzed steam pretreatment (Bura, 2004) and dilute sulfuric acid pretreatment (Lloyd and Wyman, 2005), could significantly degrade hemicellulose to monomeric or oligomeric sugars, while a large portion of lignin remains intact. On the other hand, alkaline pretreatment methods, such as ammonia fiber explosion (AFEX) (Mosier et al., 2005), modify or remove lignin efficiently but hemicellulose (Gollapalli et al., 2002). Therefore, one single pretreatment method could not reach both high degradation rate of hemicellulosce and lignin. The conclusion was drawn upon extensive tests on a vast majority of feedstocks, which may raise the concern that pretreatment results from one feedstock could not be suitable to the others. Nevertheless, several conducted comparison by various pretreatment methods on one particular biomass, like corn stover (Wyman et al., 2005a) and cotton stalks (Silverstein et al., 2007), showed similar pretreatment results.

(2) Varied severity desired to treat different components.

The term, "severity" (R_0), introduced from pulping process, was used as a rough indicator of the harsh level of pretreatment conditions, to compare the pretreatment performance. It was defined as a function of reaction time (t, min) and temperature (T, °C): $R_0 = t \cdot \exp(T-100)/14.75$ (Overend et al., 1987). The effect of pH can also be incorporated as the combined severity (CS, $CS = \log(R_0) - pH$) if pretreatment is carried out under acid conditions (Chum et al., 1990).

Among the three components of lignocelluloses, hemicellulose is most subdued to changes in pretreatment conditions, and always a low severity is sufficient to largely degrade it (Chandra et al., 2007). By contrast, higher severity will facilitate further

degradation of hemicellulose sugars to inhibitory compounds such as furfural. However, high severity is nonetheless desirable to decompose the lignin part and enhance the accessibility of cellulose by enzyme (Galbe and Zacchi, 2007). Thus, a medium severity should be employed for the compromise between efficient removal hemicellulose decomposition and delignification, and minimized over-degradation of hemicellulose sugars, but the compromised solution will sacrifice effectiveness of both functions.

Currently, the strategy to choose a pretreatment technology for a particular biomass depends on the biomass composition and target products (Hu et al., 2008). However, for most biomass types, all three components account for the major part (Kumar et al., 2009), and it would be not economically feasible to recover only one or part of the components (Hinman et al., 1989). Therefore, it is highly desirable to development a pretreatment technology featuring efficiency recovery of all major components, and negligible production of inhibitory by-products.

1.4 Multi-Stage Pretreatment

1.4.1 Process Development

In view of the above discussion that different technologies and severities should be applied for multiple purpose optimizations, a composite pretreatment streamline with separate stages was suggested. Lee et al. (1997) simply divided the dilute acid pretreatment of hardwoods into two stages with same severity at low temperature. Although the process featured a low percentage of xylose degradation and nearly no glucose degradation, the xylose and glucose yield was still fairly low due to the low applied severity. Sugar yields were even lower than the optimized one-stage process.

Torget and Hsu (1994) applied different temperatures in two-stage dilute-acid pretreatment of hybrid poplar. A low temperature (140°C) followed by a higher temperature (170°C) was employed to deal with the easy-to-hydrolyze and hard-to-hydrolyze portion of xylan, respectively, achieving a 92% xylose recovery with only 2% of xylose degraded to furfural. However, the whole process focused only on maximizing xylose yield, leaving glucan, accounting for 42% of the raw material, in a low conversion rate.

Nguyen et al. (1999; 2000) were the first to conduct a two-stage dilute acid pretreatment with separate severities, aiming to recover hemicellulose and cellulose in different stages. The pretreatment targeted the whole-tree chips), with the first stage at low severity to maximize xylose recovery and second stage under severer conditions to hydrolyze the remaining cellulose. As a result, 80-90% of hemicellulose and 50-60% of cellulose were converted to sugars throughout the pretreatment, with 93% of the rest cellulose digested by cellulase, meanwhile the usage of costly enzyme was cut by half. The highest ethanol yield could be achieved at 89%. Subsequently, continuous countercurrent extractor was used instead for the xylose recovery favoring a lower water usage with significantly reduced cost (Kim et al., 2001; Kim et al., 2002). Softwood sawdust (Kim, 2005) was also tested by the same pretreatment scheme, with a lower maximized sugar recovery (68% xylose and 52% glucose).

Söderström et al. extensively investigated two-stage steam explosion of spruce with either SO_2 (Söderström et al., 2002) or H_2SO_4 (Söderström et al., 2003b) as the supplemented acid catalyst in both steps, as well as H_2SO_4 followed by SO_2 (Söderström et al., 2003a). Higher sugar yields (77-80%) were achieved by either SO_2 or H_2SO_4 in both steps, while all three processes could lead to similar ethanol yield (59-65%).

So far, most studies of two-stage acid pretreatment focused on softwoods, which generally have high hexose content compared to pentose fraction. Therefore, the investigation needs to extend to other biomass types, with higher pentose percentage. In addition, most research mainly aimed to raise cellulose sugar recovery and its accessibility to enzyme after acid pretreatment in the second stage under harsh conditions. It would benefit from a high glucose yield and low enzyme usage, but meanwhile, inefficient lignin removal would not substantially improve the enzyme digestibility, which would not improve ethanol yield evidently. For that matter, the second stage of the pretreatment should be designed instead in a way to optimize lignin removal, and enhance cellulose susceptibility to enzymatic hydrolysis. Based on the discussion in the above section, it is clear that optimization of hemicellulose hydrolysis and lignin removal, respectively in two stages, could not be achieved by the same method or at the same severity, indicating two-stage acid pretreatment did not work out as a favorable method, neither did two-stage alkaline pretreatment (Currelli et al., 1997).

As can be concluded from Table 1.1, pretreatment favors a two-stage scheme, in which the first stage is carried out by acid-based pretreatment at low severity to decompose hemicellulose, whereas the second stage is applied at high severity by alkaline pretreatment or organosolv method for delignification. In fact, the process of an acid pretreatment followed by alkaline method emerged with the concept of biomass fractionation of over three decades back (Koukios and Valkanas, 1982). Biomass fractionation was developed as a means to improve the overall biomass utilization. It is achieved through separation of the three major biomass components prior to the refining process to obtain high value-added products, meanwhile preserved their structural and chemical integrity (Papatheofanous et al., 1995). Fractionation processes include a pretreatment process and at least one associated separation steps. Generally a dilute acid hydrolysis was applied during pretreatment to mainly recover the hemicellulose sugars, and an aqueous alkali extraction method was followed for delignification. However, throughout fractionation, the acid pretreatment was considered to be the key process to determine the fractionation yields and modification extents of components (Martinez et al., 1995). For that reason, a vast amount of studies were undertaken on acid pretreatment, rather than covering both stages (Martinez et al., 1995; Fernandez-Bolanos et al., 1999; Heitz et al., 1991; Beltrame et al., 1992).

The actual two-stage acidic-alkaline pretreatment was first applied by Maekawa (1996) for the enzymatic saccharification of a wide range of materials including rice plant, hardwoods and of softwoods. By using steam explosion and alkali-hydrogen peroxide treatment in succession, they showed results with significant improvement of enzymatic digestibility by 2-2.5 times compared with the single steam explosion scheme. The most remarkable effect was found on the treatment of softwoods. In the following decades, several more studies were reported to investigate two-stage pretreatment processes (with varied methods, some also called post-treatment) of various biomass types, which were summarized in

. A general flow diagram of two-stage acidic-alkaline pretreatment process was presented in Figure 1.3.

Most of the discussed two-stage pretreatment methods could lead to higher sugar recovery than single stage processes, and require less enzyme loading. Although the

advantages of two-stage pretreatment have been partly verified, so far the studies on two-stage pretreatment entailed essential shortages:

(1) With only 10-20 reports on two-stage acidic-alkaline pretreatment in the last two decades, the pretreatment method lack adequate and systematical studies. For one particular biomass, only one acid and alkaline pretreatment method were applied in two stages respectively. No investigation was conducted for the comparison of varied acid or alkaline treatment methods in each stage on the overall pretreatment efficiency.

(2) Most studies only focused on the recovery rate of total reducing sugars and the removal extent of lignin. Little research showed inhibitory by-products formation, and how it was associated with sugars recovery. In addition to that, lack of study on the downstream fermentation of pretreated biomass and hydrolysates could not provide in-depth understanding of the effect of two-stage processes on ethanol yield.

1.4.2 Potential Economic Favorability

As discussed previously, two-stage pretreatment resulted in higher ethanol yield and produce value-added lignin product, while required low enzymes usage. Besides, after fractionation the substrate ends up with much concentrated cellulose, thereby reduced the desired distillation energy (Kadam et al., 2009). All those benefits would lower the overall production cost. However, two-stage process is more capital and energy intensive due to the addition of a new processing unit. Therefore, a techno-economic analysis is necessary to compare the trade-offs and evaluate the economic feasibility of the process.

Techno-economic analysis has been used to assess the cellulosic biofuels production processes previously (Wyman et al., 2005b; Galbe and Zacchi, 1992; So and Brown, 1999). The approach was intensively and systematically applied at National Renewable Energy Laboratory (NREL) (Aden and Foust, 2009). Evaluation has been carried out for comparison between biochemical and thermo-chemical routes (Foust et al., 2009), and comparison among associated pretreatment technologies (Eggeman and Elander, 2005), but few was made on two-stage pretreatment processes.

Wingren et al. (2004) compared two-stage steam explosion of spruce with one step process from techno-economic standpoint. Two-stage process resulted in higher ethanol yield (74.6% compared to 71.8% from one-stage), but incurred higher capital cost and energy requirement. The economic evaluation showed the same sustained production cost for both processes. However, it has been pointed out that the cost of two-stage process could be brought down by further improved ethanol yield, no pressure release between two stages, and applying higher solid loading in the second step.

The techno-economic analysis was conducted by Kadam et al. (2009) on two-stage acidic-alkaline pretreatment of corn stover in terms of the performance at a pilot plant. The first stage was carried out by concurrent dilute acid pretreatment, and the second stage applied concurrent delignification with NaOH. The evaluation result was compared with NREL one-stage process (Aden et al., 2002). Although lower hemicellulose hydrolysis efficiency was achieved, which led to a lower end ethanol yield (58 compared to NREL 69 MGal/yr), the minimum ethanol selling price was projected to be slightly higher for the two-stage process ($2.3/gal compared to NREL $2.1/gal in 2000 dollar). The price was suggested to be furthered reduced below $1.9/gal if reducing NaOH usage and selling lignin at a higher price. The two-stage process was proved to be economically attractive and had great potential for commercial application.

Although the above two studies showed favorable potential of two-stage pretreatment, the economic feasibility was insufficiently evaluated on the process. Extensive investigation of techno-economic analysis should be conducted further on varied two-stage acidic-alkaline pretreatment processes and on other types of biomass, especially those with high lignin contents like *Miscanthus*.

1.5 Improvement of Hemicellulose Hydrolysis at Acid Stage

1.5.1 Alternative Acid Catalysts

In the two-stage pretreatment process, the alkaline pretreatment in the second stage for delignification left the biomass with little induced inhibitory compounds, while the first stage acid pretreatment suffered at least 10-20% loss of hemicellulose sugars to furfural depend upon initial solid loading. Among the acid pretreatment methods, dilute acid pretreatment, usually applying sulfuric acid, achieved the highest yield of

hemicellulose sugars, mainly xylose (van Walsum and Shi, 2004). As the catalyst, sulfuric acid facilitated further conversion of xylose to furfural. Therefore, alternative catalyst needs to be exploited in favor of acceptable xylose recovery rate.

Several mineral acids other than sulfuric acid, including hydrochloric acid (Goldstein et al., 1983), nitric acid (Luo et al., 2002) and phosphoric acid (Israilides et al., 1978), were used to replace sulfuric acid for hemicellulose hydrolysis, but none of them could improve the performance, and some even imposed safety concern (Yang and Wyman, 2008). Thereafter, exploit of sulfuric acid substitutes extended to organic acid, mainly carboxylic acid, and was able to attain improved pretreatment effectiveness. The investigation of carboxylic acids as pretreatment catalysts was discussed below, in two groups: Trifluoroacetic acid (TFA) and dicarboxylic acid.

TFA: Albersheim et al. (1967) first used TFA (2M) to hydrolyze the plant cell wall polysaccharides, and found out that compared to mineral acids, TFA yielded at least the same amount of monosaccharides with lower sugar degradation. Other than that, TFA has a low boiling point (72°C) and thus can be easily removed or reused through evaporation. Concentrated TFA was used to treat cereal straws (Fanta et al., 1984; Dong et al., 2009) and forage grass (de Ruiter and Burns, 1987), and it led to high xylose yield with little xylose decomposition and cellulose largely unaffected. Marzialetti et al. (2008) further studied biomass hydrolysis by TFA at different temperatures. It indicated at low temperature (150°C), TFA showed high selectivity for hemicellulose decomposition over xylose degradation. In contrast, at high temperature (200°C), the pretreatment generated less sugar but caused more furans formation. Furthermore, another potential advantage for TFA application is the likely very low impact of the chemical on the environment and living organisms including humans, animals, plants and microorganisms based on the current knowledge (Frank et al., 2002; Scott et al., 2005). Regarding its breakdown in the environment, TFA appeared to be stable in the aqueous phase and very resistant to degradation by either non-biological physicochemical processes or the majority microbial systems. However, potential for bioaccumulation in animals, bacteria and some aquatic plants is highly unlikely, whereas TFA can accumulate in certain terrestrial higher plants via roots uptake of water (Boutonnet et al., 1999).

Dicarboxylic acids: Dicarboxylic acids were initially applied as the catalyst alternative for cellulose hydrolysis. Previously, mineral acid and cellulolytic enzyme were the two major catalysts used to hydrolyze cellulose into glucose. Both catalysts had fatal drawbacks: enzymes were too expensive for commercial application, and mineral acids could further degrade cellulose into HMF. To overcome these barriers, Mosier et al. (2004) proposed to develop catalysts that mimic the catalyzing function of cellulolytic enzymes through biomimetic approach. The catalysts could entail both the cost advantage of mineral acid catalysts and the selectivity advantage of enzymes.

Catalytic function domain in cellulolytic enzymes has specific structure containing two carboxylic amino acids, either glutamic or aspartic acids. The two carboxylic amino acids system provides a pair of carboxylic acids housing the proton transfer from one site to the other and thereby fuels the cellulose hydrolysis (Mosier et al., 1999). Monocarboxylic acids (acetic acid), dicarboxylic acids (maleic acid, succinic acid) and even tricarboxylic acids (citric acid) were employed to mimic the enzymes as the catalysts for cellulose hydrolysis (Mosier et al., 2001; Mosier et al., 2002). It showed that like mineral acid catalysts, the hydrolysis efficiency of the applied acids correlated with their acidities. Dicarboxylic acids were more efficient than monocarboxylic acids because they are stronger acids. Among others, maleic acid was the most efficient – as effective as dilute sulfuric acid – but with minimal glucose degradation.

Hemicellulolytic enzymes have similar structures and hydrolysis mechanisms as cellulolytic enzymes (McCarter and Withers, 1994), so the biomimetic effect of maleic acid could be extended to hemicellulose hydrolysis. The acid was applied for hemicellulose hydrolysis in corn stover (Lu and Mosier, 2007). Under optimal conditions, sulfuric acid caused more than 30% of xylose degradation. By contrast, 95% xylose recovery was achieved by maleic acid hydrolysis followed by 87% of theoretical ethanol yield. Subsequent studies showed an even higher xylose yield – 96% of theoretical – at higher catalyst concentrations and lower temperatures (Lu and Mosier, 2008). The maleic acid isomer, fumaric acid, was also tested (Kootstra et al., 2009a) and showed less selectivity compared to maleic acid, probably due to the higher pK_a.

In addition to the catalysis efficiency, the recovery capability should also be taken into account as one major factor for assessment of the candidate acid catalysts. Compared

with other acids, TFA has the lowest boiling point and high evaporability, therefore can be easily recycled by esterification by reactive distillation (Mahajan et al., 2008). Recovery of sulfuric acid has been extensively studied previously and can be achieved mainly through freeze crystallization, acid retardation, and diffusion dialysis. In contrast, maleic acid is hard to remove through distillation (boiling point 135 ºC) and a mature technology still lacks for its efficient recovery.

1.5.2 Combined Acid Catalysis

Although TFA and maleic acid were found to have high selectivity for hemicellulose decomposition, their introduction may cause new problems: Evaporation of TFA increase the operation cost significantly; The cost for maleic acid catalysis was still ten times that of sulfuric acid process (Lu and Mosier, 2007). Besides, another concern may be raised of their impact on the downstream fermentation and wastewater treatment processes.

An alternative approach to mitigate the impact of TFA and maleic acid is to replace them with cheap catalysts such as commonly used sulfuric acid. Biomimetic approach has been proved to be effective by combining the cost advantage of mineral acid and the selectivity advantage of enzymes, and the production cost was driven down remarkably compared to enzymatic hydrolysis. This strategy could be applied again to further reduce the cost, but this time with mineral acid usage.

Combining mineral acid (sulfuric acid) and organic acid (TFA or maleic acid) for hydrolysis appeared to be theoretically logical. Mosier et al. (2002) concluded that sulfuric acid had the same mechanism for cellulose hydrolysis as maleic acid, but with higher hydrolysis ability. On the other hand, the two acids had distinct mechanisms during sugar degradation. For that reason, the advantage of combined acid catalysts system is perceptible: the mineral acid portion catalyzed the polysaccharide hydrolysis efficiently, while the organic acid portion disturbed the protonation of sugar and thereby prevented its degradation. Integrating the advantage of each acid, the combined catalysts could achieve improved hydrolysis efficiency than that by individual catalyst.

In fact, the combination of organic and inorganic molecules bearing acidic groups was intensively applied for asymmetric synthesis (Yamamoto and Futatsugi, 2005). The combined acid system resulted in higher reactivity, selectivity, and versatility than the use

of individual catalyst. It was also suggested that adding a second catalyst that slows down the reaction rate could add benefit by suppressing unwanted side reactions (Schreiner, 2010). Both of the above principles could be applied in the hemicellulose hydrolysis.

1.6 Objectives and Hypotheses

The goal of this project is to improve the pretreatment performance of bioethanol production through advancing in-depth understanding of the pretreatment mechanisms and developing advanced pretreatment technologies accordingly. The two specific experimental objectives outlined below were designed to meet this goal.

Objective #1: To quantitatively describe the hydrolysis of primary biomass components through two-stage acidic-alkaline pretreatment and to evaluate the pretreatment performance.

During the biofuels production, high fuel ethanol yield necessitates low degradation rate of intermediate sugars and sufficient lignin removal. However, these two chemical conversion pathways favor both high temperature and extended reaction time. Therefore a compromise among pretreatment conditions always needs to be made to reach a balance between the two targets (Galbe and Zacchi, 2007). Furthermore, efficient decomposition of biomass components was achieved by various pretreatment methods: hydrolysis of cellulose and hemicellulose were in favor of acid-based pretreatment methods, while alkaline pretreatment effectively reduced lignin content (Chandra et al., 2007). Based on the above features of biomass depolymerization, it is hypothesized that a two-stage pretreatment could achieve both high sugars recovery and low inhibitory by-products formation by applying acid pretreatment under mild conditions in the first stage and alkaline pretreatment under much harsher conditions in the following stage. Two stages function differently to mainly undertake hemicellulose hydrolysis and lignin removal, respectively. Diluted sulfuric acid pretreatment will be carried out in the 1st stage, while lime pretreatment will be conducted subsequently. The best pretreatment conditions will located through process optimization by conducting response surface methodology analysis taking account of catalyst dosage, temperature and residence time. The

pretreatment performance will be evaluated thoroughly in terms of sugars yield, by-products yield, biomass structure changes, and end ethanol yields.

Objective #2: To evaluate the effectiveness of hemicellulose hydrolysis by combined acid catalysts with sulfuric acid and biomimetic acids (carboxylic acids), and to characterize the individual function of each acid component during the hemicellulose hydrolysis.

For cellulose hydrolysis, sulfuric acid has the same mechanism as carboxylic acids, but with higher hydrolysis rate. In the subsequent process, sulfuric acid degrades glucose while carboxylic acids do not (Mosier et al., 2002). TFA has been shown to have the similar features as carboxylic acids. It has been proposed that the anion of organic acid may prevent the degradation of glucose by shielding it from protonation of hydroxyl group (Kootstra et al., 2009b). According to the analogy between the mechanisms of cellulose and hemicellulose hydrolysis (Lu and Mosier, 2007), it is hypothesized that sulfuric acid has the catalysis advantage in the hemicellulose hydrolysis, and biomimetic acids (carboxylic acids) could prevent the degradation of xylose. Further, similar to the biomimetic approach, the unique advantage of the two acid types in different hydrolysis phases could be combined to develop more efficient hydrolysis catalysts. For that matter, it is hypothesized that combined acid catalysts with a mineral acid (sulfuric acid) and biomimetic acids (carboxylic acid) could result in both high efficiency and selectivity for hemicellulose decomposition than individual acids. In the study, sulfuric acid will be blended with TFA and MA individually to form the combined acid systems. The effects of acid blending ratio, temperature and acid dosage on the hydrolysis performance will be assessed. The hydrolysis performance will be evaluated thoroughly in terms of sugars yield, by-products yield, biomass structure changes, and end ethanol yields.

1.7 References

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1.8 Figures and Tables

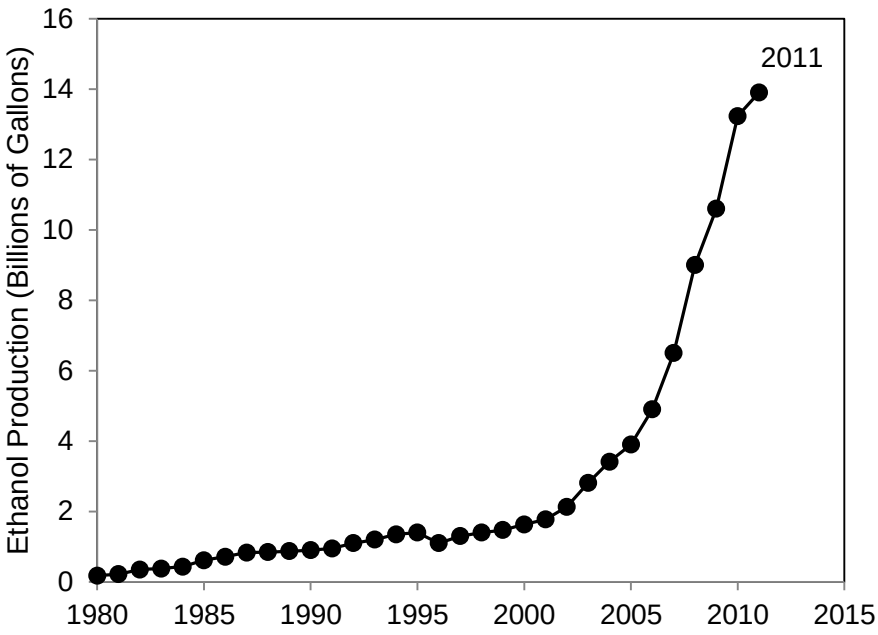

Figure 1.1. Historic U.S. fuel ethanol production. Source: Renewable Fuels Association.

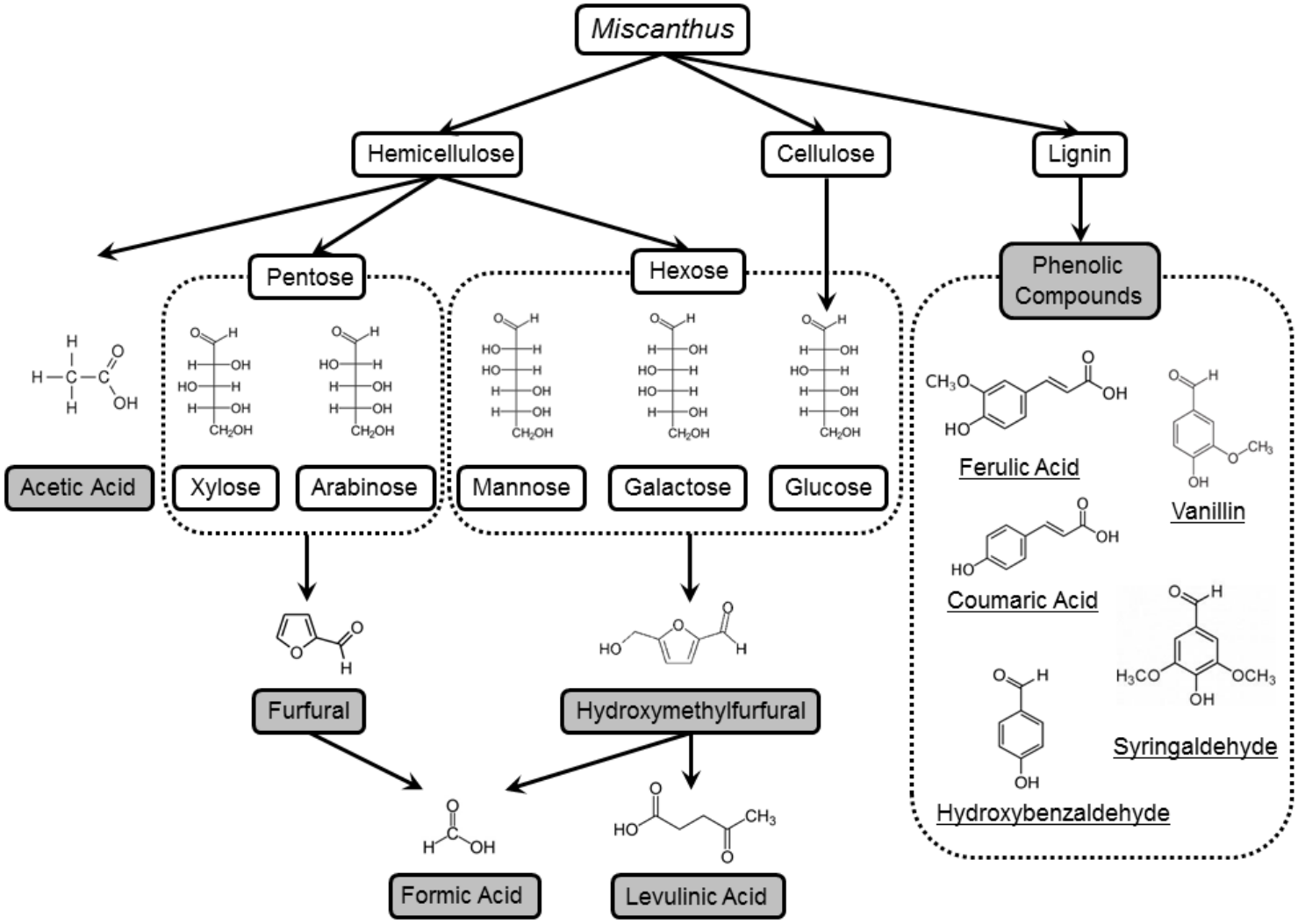

Figure 1.2. Hydrolysis pathway of lignocellulose. Grey background means the chemical is inhibitory to the fermentation process.

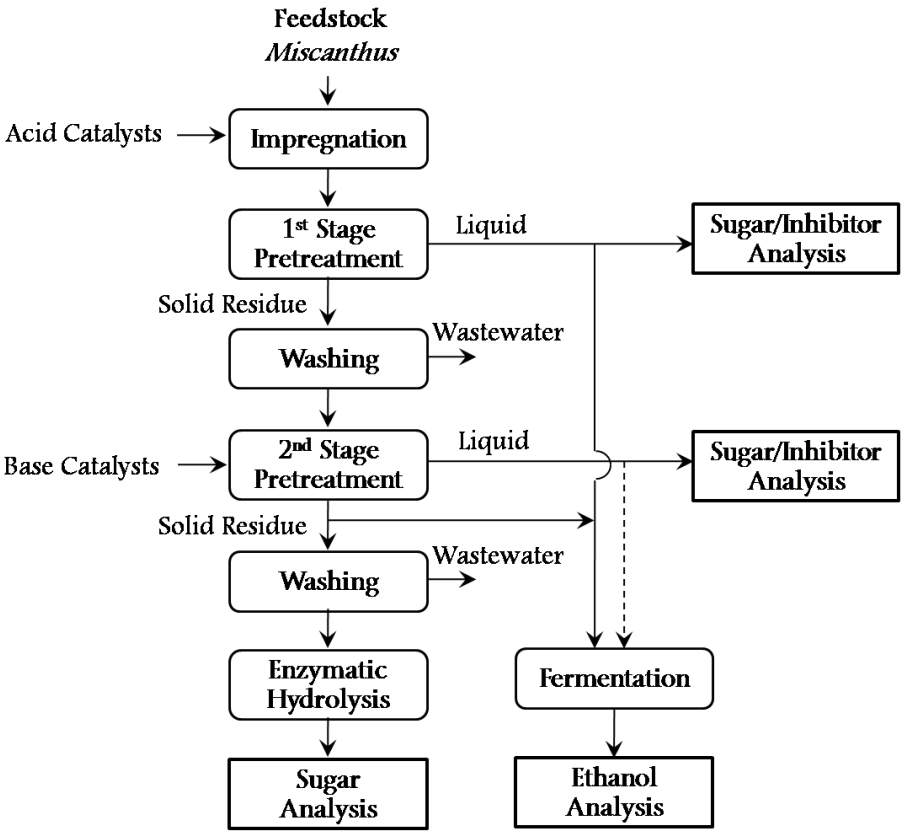

Figure 1.3. Schematic diagram of two-stage acidic-alkaline pretreatment process.

Table 1.1. List of physicochemical pretreatment technologies

Pretreatment technology	Advantages	Disadvantages
Acid-based		
Dilute acid hydrolysis	Efficient degradation of hemicellulose; some lignin disruption; Inexpensive.	Destruction of part of xylose; form fermentation inhibitors; toxic and corrosive.
Concentrated acid hydrolysis	Highly improvement of enzymatic hydrolysis.	Reactants are toxic, corrosive, hazardous; High cost.
SO_2 explosion	High removal of hemicellulose; form less fermentation inhibitors compared with steam explosion; moderate cost.	Destruction of part of xylose; limited disruption of lignin.
Supercritical CO_2 explosion	Cost effective; no formation of inhibitors; no need for neutralization.	Does not modify lignin or hemicellulose; low yield.
Neutral		
Steam explosion	High removal of hemicellulose; moderate cost; no addition of chemicals;	Destruction of part of xylose; form fermentation inhibitors; limited disruption of lignin.
Liquid Hot Water	Relatively high removal of hemicellulose; low degradation of sugars; no addition of chemicals; low need for neutralization; no need for size reduction.	Limited disruption of lignin; favor low dry matter content; higher downstream energy demand.
Ozonolysis	Effective degradation of lignin; does not produce toxic residues; no need for neutralization; room temperature and pressure.	Large amount of ozone required; expensive; low removal rate of hemicellulose.
Alkaline		
Dilute NaOH treatment	High removal of lignin;	Ineffective degradation of hemicellulose; ineffective for high lignin content biomass; irrecoverable salts formed and incorporated into biomass; low lignin recovery.

Table 1.1. (cont.)

Pretreatment technology	Advantages	Disadvantages
Lime pretreatment	High removal of lignin; Low reagent cost; safe to handle; reagent recoverable;	Ineffective degradation of hemicellulose; ineffective for high lignin content biomass; lower reaction rate compared with other alkaline methods; low lignin recovery.
Ammonia fiber explosion (AFEX)	High removal of lignin; produce less downstream inhibitors; no need for size reduction; ammonia could be reused; could handle high dry matter content;	Ineffective degradation of hemicellulose; ineffective for high lignin content biomass; high cost of ammonia and ammonia recovery; low lignin recovery.
Ammonia recycle percolation (ARP)	High removal of lignin; produce less downstream inhibitors; both efficient for hardwood and low lignin content biomass; ammonia could be used;	Ineffective degradation of hemicellulose; less effective for softwoods; high energy cost with high liquid loadings; low lignin recovery.
Wet oxidation (O_2, H_2O_2)	High removal of lignin; inhibitors can be oxidized to carboxylic acid; relatively higher degradation of hemicellulose compared with other alkaline methods	Ineffective for high lignin content biomass; low lignin recovery.
Solvent-based		
Organosolv	Both hydrolyzed lignin and hemicellulose; high lignin recovery.	High cost; necessary to remove solvents; form inhibitors from hemicellulose.
Ionic Liquids (ILs)	Can achieve high cellulose digestibility at mild conditions;	High cost; necessary to remove ILs; inadequate researches.

Table 1.2. Two-stage pretreatment technologies reported in time ascending sequence (With varied methods in each stage)

Source	Biomass	1st stage method	2nd stage method	Maximal sugar yields	Maximal ethanol yield
Papatheofanous et al., 1995 (Martinez et al., 1995)	Wheat straw	Dilute-acid pretreatment	Organosolv	94% cellulose conversion, 87% hemicellulose removed, more than 70% lignin removal	88%
Maekawa, 1996 (Maekawa, 1996)	Rice, 3 hardwoods, 3 softwoods	Steam explosion	Alkali H_2O_2 treatment	--	--
Wu et al., 1997 (Wu and Lee, 1997)	Switchgrass (35% G, 17% X, 24% L)	Two-stage dilute-acid percolation	Ammonia recycled percolation	96% glucan conversion, 83% lignin removal	--
Montane et al., 1998 (Montané et al., 1998)	Wheat straw (32% G, 21% X, 17% L)	Steam explosion	Alkali treatment	70% glucose, 55% pentose, 70% lignin recovery	--
Sun et al., 2002 (Sun and Sun, 2002)	Rice straw (37% C, 34% H, 12% L)	Ethanol – H_2O – HCl (Organosolv)	H_2O_2 treatment	88% hemicellulose, 94% lignin removal	--
Yang et al., 2002 (Yang et al., 2002)	Wood chips (48% C, 21% H, 30% L)	Steam explosion with SO_2	Alkaline peroxide treatment	82% polysaccharides recovered, 90% lignin solubilized	--
Pan et al., 2004 (Pan et al., 2004)	Wood chips (48% C, 21% H, 30% L)	Steam explosion with SO_2	Alkali-oxygen treatment	Over 90% glucose, 84% lignin removal	--
Pan et al., 2005 (Pan et al., 2005)	Wood chips (48% C, 21% H, 30% L)	Steam explosion with SO_2	Alkali treatment	85% glucose, 34% lignin removal	--

Table 1.2. (cont.)

Source	Biomass	1st stage method	2nd stage method	Maximal sugar yields	Maximal ethanol yield
Sun et al., 2005 (Sun et al., 2005)	Wheat straw (39% C, 39% H, 17% L)	Steam explosion	Alkaline peroxide treatment	88% hemicellulose degradation, 99% lignin removal	--
Kim et al., 2006 (Kim and Lee, 2006)	Corn stover (38% G, 21% X, 18% L)	Hot water treatment	Aqueous ammonia treatment	96% glucose, 86% xylose, 81% lignin removal	--
Buranov et al., 2007 (Buranov and Mazza, 2007)	Flax shives (34% G, 21% X, 30% L)	Hot water treatment	Aqueous ammonia treatment	95% hemicellulose removal, 77% lignin removal	--
Sorensen et al., 2008 (Sørensen et al., 2008)	*Miscanthus* (44% G, 21% X, 25% L)	Dilute-acid presoaking	Wet explosion	61% glucose, 95% xylose,	--
Brosse et al., 2009 (Brosse et al., 2009)	*Miscanthus* (38% G, 34% X, 25% L)	Dilute-acid presoaking	Organosolv	95% glucose, 73% xylose, 71% lignin recovery	70%

Note: G-glucose, X-xylose, C-cellulose, H-hemicellulose, L-lignin.

CHAPTER 2

TWO-STAGE ACIDIC-ALKALINE HYDROTHERMAL PRETREATMENT OF LIGNOCELLULOSE FOR THE HIGH RECOVERY OF CELLULOSE AND HEMICELLULOSE SUGARS[1]

Abstract

Sequential acid and alkaline hydrothermal pretreatment using sulfuric acid and lime were evaluated to recover hexose and pentose from biomass. Process performance was optimized in terms of catalyst concentration, retention time and temperature using Response Surface Methodology. Medium operational conditions in the acid stage and harsh conditions in the alkaline stage were desirable with optimal performance at 0.73 wt% H_2SO_4, 150 ºC, 6.1 min in the first stage, and 0.024 g lime/g biomass, 202 ºC, 30min in the second stage. In comparison to single stage pretreatments with high recovery of either glucose or xylose, two-stage pretreatment produced >80% glucose and >70% xylose yield. In addition, the method greatly improved ethanol fermentation with yield up to 0.145 g/g *Miscanthus*, due to significantly reduced formation of inhibitory by-products such as weak acids, furans and phenols. Supplementing biomimetic acids would further increase glucose yield by up to 15% and xylose yield by 25%.

Keywords

Two-stage acidic-alkaline pretreatment, *Miscanthus*, Combined acid hydrolysis, response surface methodology, lignocellulose

[1] This chapter is *in preparation for submission to Bioresource Technology*.

2.1 Introduction

Utilizing lignocellulosic biomass as sustainable material has lately become a compelling alternative among conversion technologies in the biofuels and bio-based industry. Widely distributed and largely untapped, lignocellulose can continuously provide low cost feedstock (Cardona and Sánchez, 2007), which would avoid disturbing the food supply as is the problem with conventional biofuels. On the other hand, lignocellulose derived biofuels are not yet commercially feasible, due to the associated prohibitive conversion and feedstock logistics costs (EERE, 2011). Recently, it has been noticed that the unfavorable process economics can be improved by means of efficient co-utilization of cellulose and hemicellulose instead of cellulose fraction alone which was focused in the past (Chandra et al., 2007). However, the stringent requirement of utilizing all lignocellulose components would impose great challenges on the existing conversion processes, especially the initial pretreatment step. Previously, the pretreatment process was designed with the major objective of effective cellulose recovery, and accordingly a variety of pretreatment methods has been developed including physical, chemical, physicochemical, biological methods and their combinations (Kumar et al., 2009). Unfortunately, none of these methods can also obtain high sugar recovery extensively from hemicellulose (Mosier et al., 2005).

To achieve maximum multiple sugar yields simultaneously, pretreatment streamline was suggested to be divided into separate stages (Nguyen et al., 2000; Kim, 2005). It was well known that the severity of pretreatment conditions greatly affects the hydrolysis of lignocellulose components, especially hemicellulose (Galbe and Zacchi, 2007). A sever condition would cause significant degradation of hemicellulose sugars into inhibitory compounds, while a relatively high degree of severity is still desirable to enhance the enzymatic digestibility of cellulose. Therefore, in the separate pretreatment process, varied severities were applied, where the first stage was conducted at low severity for efficient hemicellulose hydrolysis, and another stage under more severe conditions was followed to treat the remaining residue (Taherzadeh and Karimi, 2008). In addition to different severities application, distinctive pretreatment methods were conducted in each stage to further improve the overall biomass utilization. This fractionation strategy was based on an essential feature that most pretreatment methods have varied preference to

treat certain specific components. As such, acid pretreatment can be used to mainly hydrolyze hemicellulose while alkaline pretreatment to efficiently modify or remove lignin (Chandra et al., 2007). Up to date, the scheme of sequential acid and alkaline pretreatment was investigated the most. A wide range of promising pretreatment methods has been employed including dilute acid hydrolysis, steam explosion and hot water treatment in the acid stage succeeded by ammonia, alkaline peroxide treatment and Organoslov process in the alkaline stage (Pan et al., 2005; Sun et al., 2005; Kim and Lee, 2006; Brosse, Sannigrahi and Ragauskas, 2009). Many of them proved significantly improved yields of both cellulose and hemicellulose sugars, and required less enzymes for hydrolysis than single stage pretreatments.

Although the previous studies on two-stage pretreatments have verified the above shown benefits, the effect of pretreatment conditions on the production of important hydrolysis products and the overall performance was still not well known. Additionally, there was also a lack of the basic knowledge of the degradation profiles and fates for major lignocellulose components throughout two-stage processes. All these absent information would be necessary for in-depth understanding of pretreatment mechanism and further process improvement of two-stage methods.

To bridge the knowledge gap, in this study, ACidic-ALkaline pretreatments in succession (ACAL pretreatment) were developed. The two-stage process was carried out with acid pretreatment at low severity in the first stage mainly for hemicellulose hydrolysis, and then obtained efficient lignin removal and greatly enhanced cellulose digestibility in the second stage via alkaline pretreatment at elevated severity level. To make the process more commercially feasible, commonly applied dilute acid and lime pretreatments were utilized in each stage, respectively. The process was optimized by using Response Surface Methodology (RSM) analysis. Finally, under the optimal conditions, two-stage acidic-alkaline pretreatments were compared with single stage acid and alkaline pretreatments in terms of pretreatment effectiveness. The objective of this study was to evaluate the influence of major pretreatment conditions on ACAL process, quantitatively characterize the biomass components degradations, and clearly identify the advantages of ACAL process over single stage pretreatments.

2.2 Materials and Methods

2.2.1 Raw Material

Miscanthus was used in this research as the model feedstock. The material was harvested in spring 2008 on the farm in Urbana, IL., and then air dried below 45 ºC to obtain a dry matter content between 91-94%. The dried material was hammermilled, and the fraction passing through ¼-in. (6.35mm) sieve was collected and analyzed for its contents of major components according to the NREL standard procedures (Technical Report NREL/TP-510-42618). The chemical composition of the dry based *Miscanthus* was 39.2±0.3% glucan, 19.5±0.4% xylan, 1.2±0.1% arabinan, and 24.2±1.1% lignin.

2.2.2 Pretreatment Setup and Operation

In the first stage of acid pretreatment, experiments were carried out in a batch reactor (Model 4534, PARR Instrument Co., Moline, IL) equipped with 2 L cylindrical pressure vessel (9.5 cm i.d.). 120 g of dry based *Miscanthus* samples were loaded for each batch with various acid solutions to keep a fix solid loading of 20% by weight. The pretreatment applied pure sulfuric acid solutions, and sulfuric acid solutions mixed with biomimetic acids individually. The biomimetic acids applied in this study were trifluoroacetic acid (TFA) and maleic acid (MA). Preceding the reaction in the vessel, the biomass was steeped in the acid solutions for 9 h at ambient temperature. After loaded with the reactants, the vessel was clamped shut and then heated at 6-8 ºC/min. Counting of the reactions was started once the vessel reached the desired temperature, and the vessel was controlled at a constant temperature and pressure with agitation at 400 rpm. Once the pretreatment finished, the system was cooled down to 60 ºC in about 10 min and the pressure was released immediately thereafter. After completion of the acid pretreatment, the solids and liquids were separated through Whatman No.1 filter paper. Hydrolysates (liquid fractions) were stored for chemical analysis and further use in the fermentation tests. Solid residues were air dried at 37 ºC till reaching 90-95% dry matter contents and then used in the 2nd stage alkaline pretreatment.

A different batch reactor (Model 4593, PARR Instrument Co., Moline, IL) was set up for the 2nd stage pretreatment with 100 mL cylinder-shaped pressure vessel (3.3 cm i.d.). The operation procedure of the reactor was the same as that of the acid pretreatment

reactor. Differently, 6g of dried solid residues from 1st stage pretreatment were loaded with lime solution to bring the solid loading to 20% by weight. After the 2nd stage reaction, the reacted biomass was filtered and the liquid fraction was collected for chemical analysis. Solid residues were tested for enzymatic digestibility and blended with 1st stage hydrolysates accordingly for fermentation tests.

2.2.3 Experimental Design and Statistical Analysis

The central composite design (CCD), which is the standard Response Surface Methodology (RSM), was applied in both stages separately for optimization of the pretreatment conditions. In the acid stage, acid dosage, temperature and residence time were taken as the independent variables, since it has been found that the process chemistry during hemicellulose hydrolysis greatly depended on these three factors (Shatalov and Pereira, 2011). On the contrary, it has been observed that during the lime pretreatment at temperature higher than 80 ºC, retention time had little effect on glucose yield if longer than 30 min (Saha and Cotta, 2008; Rabelo, Filho and Costa, 2009). Therefore in the optimization study of the 2nd stage, only lime loading and temperature were selected as two independent variables, with a fixed retention time of 30 min. Each independent variable at both stages was investigated at five levels. The variables were coded at the beginning to exclude the effect of their individual values under different units. The ranges and levels of the variables were given in Table 2.1. Yields of sugars (glucose and xylose), furans [furfural and hydroxymethylfurfural (HMF)], weak acids (acetic, formic and levulinic acids) and total phenols were selected as the response variables, respectively. The response variables were approximated by a two-order Taylor expansion:

$$y = \beta_0 + \sum \beta_i x_i + \sum \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 + e \qquad (1)$$

Where y is the predicted response, x_i and x_j are coded values of the independent variables, β_0, β_i, β_{ij} and β_{ii} are the Taylor expansion coefficients, and e is the error of the fitted model.

The regression and statistical analysis were carried out using Microsoft Origin 8.0, and the visualization of response surfaces were displayed by MATLAB 7.13.

For furans and weak acids which contain multiple responses, a composite response surface was derived to locate the best compromise among the responses through desirability function approach (Carlson and Carlson, 2005). In this approach, all the related responses were weighed together into one criterion, an overall desirability function, which was then optimized by RSM. The overall desirability (D) was calculated as a geometric mean of all individual desirabilities (d_i) by different weight depending on its importance to the response as follows:

$$D = (d_1^{w1} \times d_2^{w2} \times \ldots \times d_m^{wm})^{1/(w1+w2+\ldots+wm)} \quad (2)$$

Where wi $(1 \le i \le m)$ is the weight factor for each desirability. In the study, we assumed all related individual by-products (furans and weak acids) contributed equally to the overall adverse effect on fermentation, and their own inhibitory effect was employed to interpret the individual desirability (Pinzauti et al., 1996).

In this work, most pretreatment tests and all fermentation experiments were carried out in duplicate, while enzymatic hydrolysis was performed in triplicate. A 95% confidence level was applied for data analysis.

2.2.4 Enzymatic Hydrolysis

The Pretreated solid materials were enzymatically hydrolyzed following the NREL standard procedure (Technical Report NREL/TP-510-42629). Hydrolysis was conducted in 50 mM sodium citrate buffer (pH 4.8) at the loading of 1.0 wt% glucan content. Applied enzyme loadings were 15 FPU/g glucan of cellulase (Spezyme CP, Genencor), 2 CBU/FPU of β–glucosidase (Novozym 188, Sigma-Aldrich) supplemented with xylase (Multifect Xylanase, Genencor). The test flasks were incubated at 50 ºC for 72 h, and hydrolysates were sampled every 24 h.

2.2.5 Simultaneous Saccharification and Co-Fermentation (SSCF)

S. cerevisiae DA2416 was used as the host strain for producing ethanol from xylose and glucose in the pretreated hydrolysates. Methods for strain cultivation were described previously (Ha et al., 2011). SSCF was carried out in 250 mL flasks containing 50 mL of YP (1% w/v yeast extract, 2% w/v peptone) with pretreated *Miscanthus* slurry including solid residue and hydrolysate (10% w/v solid loading) at 30 ºC and 100 rpm. The initial

pH of medium was adjusted to 5.0 ± 0.1 through overliming (addition of $Ca(OH)_2$ to pH 10-11 first, followed by H_2SO_4 down to pH 5). Yeast was inoculated with an initial cell concentration of 0.35 g/L. During SSCF, Spezyme cellulose cocktail (30 FPU/g hydrolysate), Novozyme 188 β-glucosidase (60 CBU/g hydrolysate) and Multifect xylanase (0.25 mL/g hydrolysate) were supplemented for saccharification of hydrolysate. After 48 h of SSCF, newly cultured cells (0.35 g/L) were added in order to enhance sugars consumption.

2.2.6 Analytical Methods

For pretreatment and enzymatic hydrolysis tests, the concentrations of monosaccharides, furans, weak acids were measured using a HPLC system (Shimadzu) equipped with a refractive index detector (Waters) as described previously (Guo et al., 2012). Oligosaccharides in the hydrolysates were broken down to monosaccharides through 4% w/w sulfuric acid hydrolysis at 121 ºC for 60 min for quantitative analysis by HPLC. Hydrolysates after pretreatment were analyzed for phenolic compounds by GC/MS system according to previously reported methods (Guo et al., 2012). Prior to the analysis, hydrolysate samples were extracted with ether twice at 3:1 and subsequently the ether phase was concentrated by nitrogen bubbling. In addition, total phenols of the hydrolysates were determined using the Folin-Ciocalteu assay (Scalbert, Monties and Janin, 1989). Samples were diluted by water to adjust absorbance in 0.1-0.5, and total phenols were expressed in gallic acid equivalent.

For fermentation tests, glucose, xylose, xylitol, glycerol, acetate, and ethanol concentrations were determined by HPLC system (Agilent Technologies 1200 Series) equipped with a refractive index detector using a REzex ROA-Organic Acid H^+ (8%) column (Phenomenex Inc., Torrance, CA). The column was eluted with 5 mM sulfuric acid at 0.6 mL/min at 50 ºC.

All the chemicals used in the study were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (St. Louis, MO).

In the acid pretreatment, the combined severity factor (CSF) was used to describe the severity level of the pretreatment conditions taking account of the effects of reaction time,

temperature and acid dosage (Brosse, Sannigrahi and Ragauskas, 2009). The CSF was defined as:

$$CSF = \log\{t \exp\lfloor (T - T_{ref})/14.7 \rfloor\} - \mathrm{pH} \quad (3)$$

where t was hydrolysis time in min, TH was temperature in ºC, T_{ref} was the reference temperature ($T_{ref} = 100°C$), and pH was the acidity of the prehydrolysates.

2.2.7 Environmental Scanning Electron Microscope (ESEM)

Miscanthus samples with/without pretreatments were first air dried below 45 ºC and then examined under ESEM (Philips XL30 ESEM-FEG, FEI Company, Eindhoven, Netherlands). Before being sent to ESEM for imaging, the dried particles were mounted on stubs and sputter coated with gold/palladium for 70 s by a Desk II TSC turbo-pumped sputter coater (Denton Vacuum, Moorestown, NJ).

2.3 Results and discussion

2.3.1 The Effect of Pretreatment Conditions on Acid Stage Performance

The contour plots of xylose, furans and acetate, and total phenols in relation to the coded values of three independent variables (acid dosage, temperature and residence time) were visualized in Figure 2.1 and constructed on the basis of fitted quadratic models. The shapes of displayed three-dimensional isocontour surfaces can be understood by combining the commonly plotted two-dimensional response surfaces with the canonical analysis results, and the isocontours described the straightforward interactions among three variables (Carlson and Carlson, 2005).

As can be observed in Figure 2.1(a), the isocontours of solubilized xylose yield described a score of partial concentric elliptic shells. Under mild conditions, xylose yield increased with all three variable values, but further raising the levels of operational variables into harsher conditions would result in evident drop in xylose yield. General ranges of acid dosage in 0.8-1.0 wt% sulfuric acid, temperature 145-155 ºC and residence time less than 30 min were desirable to maximize the xylose recovery during the pretreatment. The optimal conditions can be achieved at the center of ellipsoids (coded values of 0.58, -0.58, -0.97) at 0.90 wt%, 151 ºC and 15.3 min with maximal xylose

recovery of 13.9% dry biomass (62.5% theoretical). These conditions were comparable to the optimal ranges of 0.9-1.8 wt%, 140-153 ºC, and 6-40 min by dilute acid pretreatment on various biomass in other reports (Shatalov and Pereira, 2011; Kim et al., 2011a; Lloyd and Wyman, 2005; Kim et al., 2011b), although xylose yield was lower than those reported 76-93% theoretical, probably in favor of their lower applied solid loading (5-7%). In addition, the optimal combined severity factor (1.7) was also lower than 2.0-2.3, the only reported value by dilute acid pretreatment on *Miscanthus* (Guo et al., 2008). Besides, Figure 2.1(a) also presented that the ellipsoids were elongated along the axis of residence time, which indicated less influence of time on xylose yield than the other two parameters. Here glucose yield was not taken in account for the process optimization in acid stage, since the primary target was hemicellulose hydrolysis to xylose.

Figure 2.1(b) showed contour plots of composite response surface through desirability function approach integrating acetic acid, furfural and HMF yields. All three hydrolysis by-products would exert evident inhibitory effects on the downstream fermentation. However, at the induced concentration in this study (3.8-15.2 g/L acetic acid, 0.9-13.2 g/L furfural, 0-3.0 g/L HMF), acetic acid presented the greatest inhibition. Additionally, the formation of three by-products increased with pretreatment severity, although as for acetic acid it tended to level off at higher severity level (data not shown). Based on the different inhibitory effects of furans and acetic acid, the impact of furans changed remarkably at greater presence, while that of acetate moved faster at low concentration. When taking account of concentration and individual effect, the composite contour plots described steadily decreasing overall desirability as severity level increased, which meant continuously intensifying inhibitory effects. At low severity, acetic acid contributed the most to the overall desirability change whereas furans took over at high severity. In addition, the isoresponse contour surfaces tuned parallel to the axis of residence time while above 25 min. This implied that any extended reaction time would not significantly affect the hydrolysis after 25 min pretreatment.

Apart from furans and weak acids, a wide range of phenolic compounds formed from lignin breakdown and carbohydrate degradation during acid hydrolysis, most of which were considered potential fermentation inhibitors as well. Total phenols under various conditions were illustrated graphically in Figure 2.1(c), and the isocontour defined a

group of curved surfaces along the axis of residence time. It can be observed that more phenols were generated with increase of operating severity, which suggested harsh pretreatment conditions were inductive to phenols formation. Besides, similarly as in cases of xylose, furans and acetate, the effect of reaction time on total phenols appeared to be trivial as can be concluded from the observed parallel surfaces along the direction of residence time. Individual phenols were also analyzed for further understanding of phenols production. Table 2.2 listed the major individual phenols with concentrations greater than 10 mg/L in the hydrolysates. Among the eight primary phenolic compounds, p-coumaric acid, ferulic acid and vanillin constituted the largest fractions. P-coumaric and ferulic acids are the primary block linkage components in herbaceous plants like *Miscanthus*, and the rest are three phenolic aldehydes along with their corresponding carboxylic acids. In fact these three aldehydes came from the three basic monolignol units in biomass individually, with p-hydroxybenzaldehyde from p-hydroxyphenyl (H), vanillin from guaiacyl (G), and syringaldehyde from syringyl (S) moiety (Klinke, Thomsen and Ahring, 2004), and this phenols profile was consistent to the biomass composition. It is also important to note that the influence of operational conditions on individual phenols varied. For most phenols, harsh conditions would induce their production, and this was in line with the trend of total phenols. By contrast, concentrations of syringaldehyde, p-coumaric and ferulic acids decreased with increase of severity levels. It was possibly due to the fact that these phenols were further oxidized to carboxylic acids and subsequently broken into smaller phenolic units. They were more reactive and served as reaction intermediates since the attached hydroxyl and methoxy group to the aromatic ring could activate the aromatic ring by electron donation (Klinke et al., 2002).

Up to date, the influence of operational conditions on the performance of dilute acid pretreatment has been intensively studied, but most of them only focused on sugar recovery (Shatalov and Pereira, 2011; Kim et al., 2011b; Akpinar et al., 2011; Cai et al., 2011). Several studies reported on furans and acetate productions, with limited information provided on the effect by single pretreatment parameter (Jeong et al., 2010; Wang et al., 2011), while there was no report on phenols yield. Here the effect of pretreatment conditions on xylose, furans, acetate and phenols were described and their

interactive tendencies can be observed when all three graphs in Figure 2.1 were put together. It clearly indicated the conditions for maximal xylose yield were not the best pretreatment conditions overall due to strong induction of most inhibitory compounds. In fact, the operational severity leveraged the reaction favorability between hemicellulose decomposition and xylose degradation. Employment of concentrated acid and elevated temperature may provide an acidic environment that accelerates formation of furfural from xylose and induces pyrolysis of lignin into phenolic compounds (Kim et al., 2011a). In this regard, medium severities would be suggested to obtain acceptably high xylose yield as well as reduced by-products formation that facilitates the xylose fermentation as a whole. In this study, the best pretreatment conditions were located at 0.73 wt%, 150 ºC, and 6.1 min. Under these conditions, the pretreatment assured 12.5% of xylose yield (56.3% theoretical), and achieved by-products formation of 1.95 g/L furfural, 6.02 g/L acetic acid and negligible HMF. Furthermore, residence time was found to have little effect on all major products production, so it could be consider least in the further process development of acid pretreatment.

All the quadratic models were tested for adequacy by the analysis of variance (ANOVA). They were highly significant and the coefficients of determination (R^2) were all above 0.9. The chosen optimal conditions were confirmed by pretreatment tests with variances of all major product yields less than 5% compared to the model predicted values.

2.3.2 The Effect of Pretreatment Conditions on Alkaline Stage Performance

Under the recommended condition for acid pretreatment, the effectiveness of post-lime pretreatment was evaluated through a 2^2 central composite design, and the response surfaces of major products were illustrated in Figure 2.2.

As shown in Figure 2.2(a), glucose release after enzymatic hydrolysis was mainly affected by temperature but lime loading. Along with temperature increase, glucose yield first increased but then declined. On the other hand, at higher temperature, glucose release was facilitated as lime loading was raised, while the opposite tendency was observed at lower temperature. High glucose yield of 0.4 g/g residue can be attained at nearly all applied lime loadings if medium temperature range of 185-220 ºC was applied.

Contrarily, the profile of weak acids in Figure 2.2(b) was simple. The overall desirability reduced continuously with both lime loading and temperature, which means generally more acetic, formic, and levulinic acids were induced from the release of acetyl group during hemicellulose and furans degradation. Through the hydrolysis, great presences of acetic and formic acids were detected, with concentrations of 4.9-9.4 g/L and 1.6-10.3 g/L, respectively (levulinic acid 0.3-0.6 g/L in contrast). It was important to note that in contrary to the primary trends shown in this figure, formic acid formation decreased to varied extent when temperature was raised up. As for the case of furans shown in Figure 2.2(c), the overall desirability was affected strongly at low lime loading levels. In fact, the inhibitory effect of furans was mainly attributed to HMF due to its high concentration in the hydrolysis (up to 3.1 g/L). HMF formation accelerated at high temperatures, especially with low lime loading. However, interestingly, HMF accumulation reduced with more lime used in the pretreatment but leveled off at high lime loading. Putting three plots together in Figure 2.2, we can conclude that similarly as in hemicellulose hydrolysis under acid conditions, during lime pretreatment, raising temperature could facilitate cellulose hydrolysis but high temperature noticeably further degraded glucose to other by-products. However, lime could slow down the latter unwanted side reaction to certain extent. Besides, the remained hemicellulose after acid pretreatment would not only be hydrolyzed to xylose but mostly further to formic acid.

Primary phenolic compounds generated through lime pretreatment were listed in Table 2.2 along with their concentrations. It has been found that the phenols present in hydrolysates were strongly dependent on the pretreatment type (Klinke, Thomsen and Ahring, 2004). For that matter, occurrence of different phenols in lime treated hydrolysates was noted in comparison with previous acidic hydrolysates. As a result, all phenols produced through acid pretreatment but ferulic acid was found during lime pretreatment. Further, lime pretreatment generated some unique phenols like syringol and methylhydroquinone. Among the detected phenols, vanillin and syringol were the most abundant. In addition, most phenols through lime pretreatment were lignin blocks with more complicated structure, which suggested that alkaline pretreatment led to incomplete lignin breakdown compared to acid pretreatment. We can also learn from Table 2.2 that generally higher operational severities could induce more phenols production.

It has been found that high glucose recovery can be obtained under two conditions during alkaline pretreatment, either long pretreatment time and low temperature, or high temperature for a short time (Wang and Cheng, 2011). Previously, alkaline pretreatment was commonly employed at lower temperatures (50-130 ºC) for extended times on the order of hours, to avoid the great loss of hemicellulose. In this work, most hemicellulose was removed in the prior acid stage, so lime pretreatment can be explored at temperatures above 170 ºC with much shortened reaction time, more favorable from an economic perspective. In fact, the applied temperatures were even higher than the previous stage to attain elevated severities for enhanced biomass susceptibility to enzymatic hydrolysis. Similar as in dilute acid pretreatment, little was known about the effects of pretreatment conditions on lime pretreatment performance especially their interactive effects (Rabelo, Filho and Costa, 2009; Fuentes et al., 2011). Other than that, since acid pretreatment was applied ahead, different profiles after lime pretreatment could be expected in this case. Indeed, only small amount of lime was necessary and there was different effect of applied temperature at high levels on glucose recovery. Normally a lime loading of up to 0.1 g/g of dry biomass was recommended in terms of high sugar recovery (Wang and Cheng, 2011; Chang, Nagwani and Holtzapple, 1998), but the amount needed was reduced to as low as 0.01 g/g in the current study. Apparently lime appeared to be more active at elevated temperature to disrupt the cellulose crystallinity and increase the biomass porosity. Meanwhile, on the flip side, enhanced lime activity also meant calcium ions could easily interact with lignin and carbohydrates with high affinity and thus impact glucose release (Torre, Rodriguez and Saura-Calixto, 1992), implying redundant lime addition was of no benefit. It can be demonstrated by the noticeable decline of glucose yield with increased lime loading at low temperatures in Figure 2.2(a). In addition, at elevated temperatures, significant drop of glucose yield occurred from its degradation, which was not observed at mild temperatures. Stripping off most hemicellulose and significant alternation of the lignocellulose structure prior to alkaline stage would also cause the cellulose sensitivity to the temperature.

For the optimization of lime pretreatment, when taking account of the four major groups of products (sugars as glucose, weak acids, furans, phenols), a compromise was made and the best conditions were located at 0.024 g/g biomass of lime loading and 202

ºC. Under these conditions, glucose yield was among the highest (78.2% theoretical) with generally lower acetic acid, furans and phenols production, as discussed in the following section.

2.3.3 Fates of Lignocellulose Components

To provide perspective into the process mechanism for ACAL pretreatment, a holistic view of the fates of primary degradation products and their distribution in the system would be necessary. Therefore, sugar degradation products from cellulose and hemicellulose were measured and presented in Figure 2.3. In addition to ACAL, single stage dilute sulfuric acid and lime pretreatments were carried out individually under their own best conditions, for comparison purpose. Moreover, our previously study (Guo et al., 2012) showed the combined biomimetic and inorganic acids could substantially improve the hemicellulose hydrolysis and recover more xylose. Thus the combined acid catalysts with trifuoroacetic acid (TFA) and maleic acid (MA) were introduced into ACAL to assess the pretreatment efficiency of the integrated process. All the tested pretreatment schemes were described in Table 2.3.

For cellulose degradation products, as can be observed from Figure 2.3(a), single acid pretreatment (P1) left considerably more cellulose intact than the other pretreatment schemes. It verified that acid catalysts were not efficient in glucose recovery. In contrast, ACAL (P3) led to nearly the same profile of cellulose degradation products as single alkaline pretreatment (P2). Recovered glucose mainly came from the treated residue after enzymatic hydrolysis, indicating the 2^{nd} alkaline stage played the key role for glucose recovery. When combined acid catalysts were adopted in the ACAL (P4/P5), glucose recovery was further improved by 8-23%. On the other hand, for hemicellulose degradation products shown in Figure 2.3(b), the profile of ACAL was similar to single acid pretreatment instead. As was reported previously, lime pretreatment would be ineffective for hemicellulose decomposition (Galbe and Zacchi, 2007). But unexpectedly here, nearly all the degraded hemicellulose went directly down to furfural. It appeared that lime was more efficient in catalyzing xylose degradation than hemicellulose decomposition, although further work was required for verification. On the contrary, single acid pretreatment could convert most hemicellulose into xylose. However, since an

elevated severity was applied as not to lose much glucose, a fair amount of xylose was inevitably degraded to furfural at the same time. ACAL could achieve efficient hemicellulose decomposition, primarily in the acid stage. Meanwhile, the separate pretreatment in ACAL allowed a low severity application in the acid stage and ensure higher xylose recovery. Similarly as for cellulose profile, introduction of combined acid catalysts in ACAL could obtain higher xylose yield through thorough conversion of oligomeric xylose.

Along with degradation of sugar polymers, lignin degradation during pretreatment was examined and the individual and total phenols under various pretreatment schemes were summarized and compared in Table 2.4. Single lime pretreatment caused substantial accumulation of total phenols, higher than single acid pretreatment by over 30%, conceivably originating from great presence of unique complex lignin derived intermediates such as syringol and hydrocinnamic acid. Compared with both single stage pretreatments (P1 & P2), ACAL could lead to significantly reduced accumulation of most phenolic compounds. Since the severity was lowered in acid stage, much less phenols were generated during acid pretreatment. In the meantime, it was also interesting to note that lignin with most hemicellulose removed appeared to be more stable during lime pretreatment. Among generated phenols, p-coumaric acid and vanillin were present with the highest concentration. When combined acids were introduced in ACAL, phenols production was further inhibited. It seems delignification was partly avoided, as can be seen as another positive synergistic effect between H_2SO_4 and biomimetic acids. Further inquiry was needed to clarity the mechanism of lignin degradation prevention in the two stage processes.

The degradation of lignocellulose components were also examined in terms of material flow balance throughout the entire process. Figure 2.4 illustrated the ACAL process with combined MA pretreatment. Mass balance was calculated in the way suggested by Percival Zhang et al. (Percival Zhang et al., 2009). Most xylan in the biomass was efficiently removed in the form of xylose in the acid stage with low degree of severity. By contrast, the following lime pretreatment managed to enhance the glucose susceptibility to enzymatic hydrolysis and obtained high recovery rate. However, lignin mostly stayed in the biomass in both stages but did not exert great adverse effect on

hydrolysis and fermentation steps. Delignification was not observed, especially in alkaline stage. One plausible explanation was that calcium ions extensively crosslinked lignin molecules and thus prevented lignin solubilization. Meanwhile, calcium also crosslinked carbohydrates, protecting them from unwanted degradations. Therefore, the situation with high lignin content was also able to avoid poor enzymatic digestibility only if the biomass porosities were effectively improved (Xu et al., 2010). From the illustrated materials flow in the figure, in total 18.4 g xylose and 39.9 g glucose could be attained after the proposed pretreatment scheme on the basis of 100 g feedstock.

2.3.4 Overall Pretreatment Effectiveness

The overall performance of two-stage acidic-alkaline pretreatment was scrutinized and compared with other tested pretreatment alternatives in terms of biomass structure alteration, sugar yields and ethanol yields.

Changes of *Miscanthus* particles were examined at an ultrastructural level. Since *Miscanthus* contains different plant fractions, here we only focused on the grinded stalk portion in each stage, and the microscopic observations were presented in Figure 2.5. By visual inspections among the stalk structures in high-resolution images, after 1st stage sulfuric acid pretreatment, the stalk was simply snapped with partial breaks of the structure, but the particle surface stayed nearly unaltered and relatively smooth. In contrast, combined acid pretreatment resulted in more disrupted structure as shown at the cross section, and rougher surface with irregular pores and cracks appeared on cell walls. It verified a more efficient decomposition of lignocellulosic structure. After 2nd stage lime pretreatment, structure disorder were further deepened with enlarged pores and hollows. In certain part of the particles, the appearance of completely disrupted surface suggested the effective disruption of the plant structure undergone through the sequential pretreatments with acid and alkali.

As can be seen from Figure 2.6, all two-stage pretreatments could achieve high yields of both glucose and xylose (at least 81% and 68% of theoretical individually). Glucose mainly came from solid residue through enhanced enzymatic hydrolysis while xylose from acid hydrolysate. ACAL with combined acids could further improve xylose yield up to 85%, apparently because most oligomeric intermediates were completely

hydrolyzed. Further, as was pointed out previously, ACAL with combined MA pretreatment could facilitate cellulose hydrolysis with enhanced glucose recovery up to 91%.

Finally, the sugar enriched residues after pretreatments were enzymatically hydrolyzed and fermented by engineered *S. cerevisiae* in a single step, with the ethanol yields shown in Table 2.5. Throughout SSCF, pH was not controlled and decreased slightly to 4.6-4.7. Both single acid and alkaline pretreatment ended with very low ethanol yield, mostly due to inefficient fermentation with only 10-20% of theoretical ethanol yield, which was noticeably less than normal. During the SSCF, the ongoing reactions ended up with steady accumulation of soluble sugars, implying the great presence of phenols and furans significantly disturbed the fermentation process but enzymatic hydrolysis. However, acetate formed through SSCF should not be counted for the inhibition since the occurring level by these single stage pretreatments was even lower than that by two-stage processes. In contrast, for sequential pretreatments schemes, ethanol yields were appreciably higher (57-63%) which reflected their benefit of less inhibitory by-products induction. Regarding substrate uptakes, glucose was rapidly consumed at the beginning, whereas overall xylose uptake rate was relatively low (less than 52%). It indicated the genetic modified yeast still needed further improvement to withhold harsh fermenting environment and reach desirable xylose consumption rate. Additionally, the considerable accumulation of acetate through SSCF might also contribute a lot to the perceived inhibitory effects. Acetate concentration in the hydrolysates after two-stage pretreatments was raised from initial 2.9-3.6 g/L up to 6.8-10.5 g/L. Among tested two-stage process, the scheme applying combined MA catalysts achieved the highest ethanol yield of 15.9 g/L, corresponding to a high yield of total reducing sugar of 65.5 g/L.

2.4 Conclusions

Pretreatment with successive acidic and alkaline stages (ACAL Pretreatment) allows to achieve high recovery of both glucose (>80%) and xylose (>70%) from biomass. Xylose was the main product in the acid stage, while glucose was recovered through lime pretreatment. In addition, the production of weak acids, furans and phenols was

remarkably reduced. The best performance could be achieved with medium severities in the acid stage and high severities in the alkaline stage. Integration of combined acid catalysts and ACAL could further improve both sugar yields and reduce primary by-products formation, with ethanol yield of up to 0.145 g/g *Miscanthus*.

2.5 Acknowledgement

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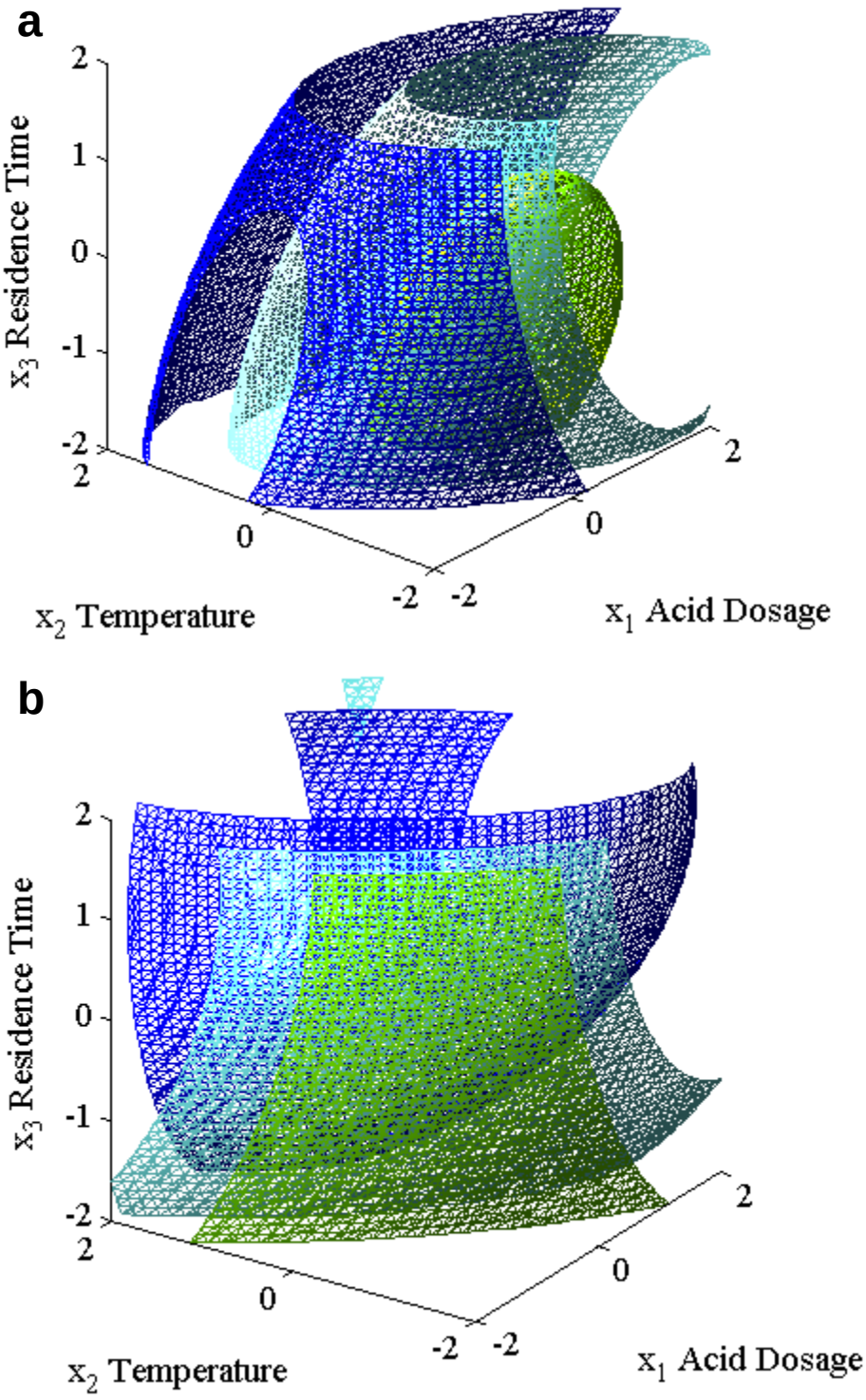
2.7 Figures and Tables



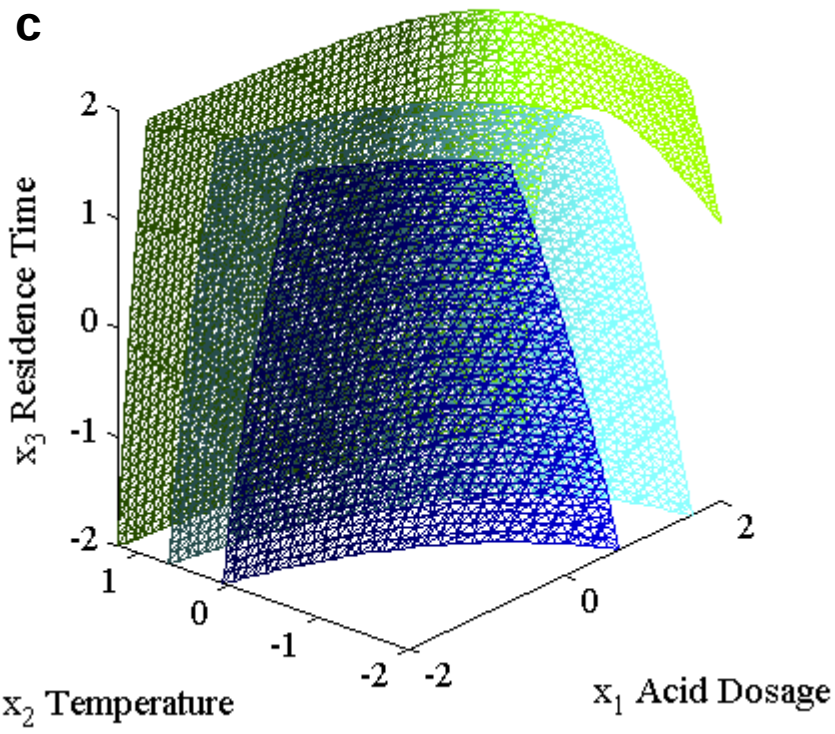


Figure 2.1. Contour plots of response surfaces in 1st stage acid pretreatment as a function of acid dosage (x_1), temperature (x_2), and residence time (x_3). (a) Xylose yield in % dry biomass. Isovalues of the isoresponse contour surfaces: 7.0% in blue, 10.0% in cyan, 13.0% in green. (b) Overall desirability of furans and acetate. Isovalues: 0.1 in blue, 0.3 in cyan, 0.5 in green. (c) Total phenols in g/L in gallic acid. Isovalues: 2.5 in blue, 3.0 in cyan, 3.5 in green.

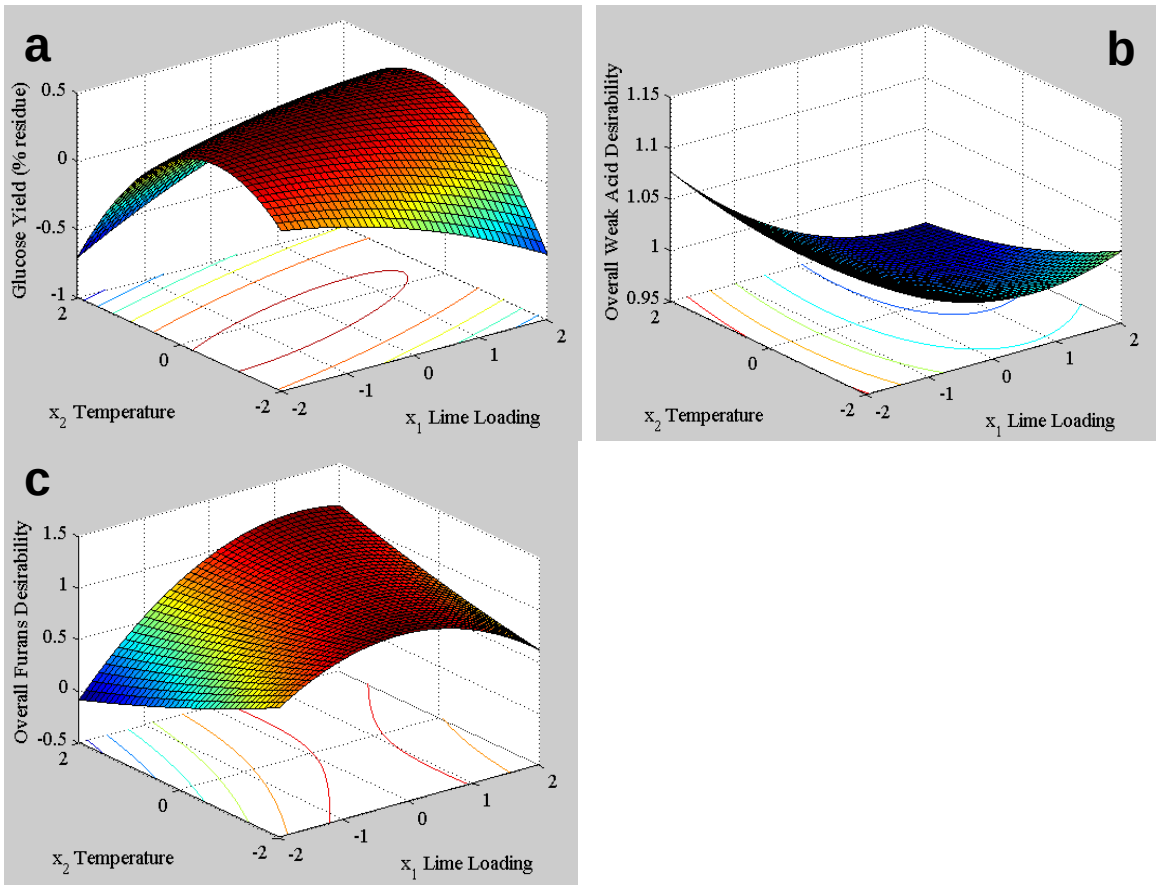


Figure 2.2. Response surfaces and contour plots in 2nd stage alkaline pretreatment as a function of lime loading (x_1) and temperature (x_2). (a) Glucose yield; (b) Overall weak acid desirability integrating acetic, formic, and levulinic acids; (c) Overall furans desirability integrating furfural and HMF.

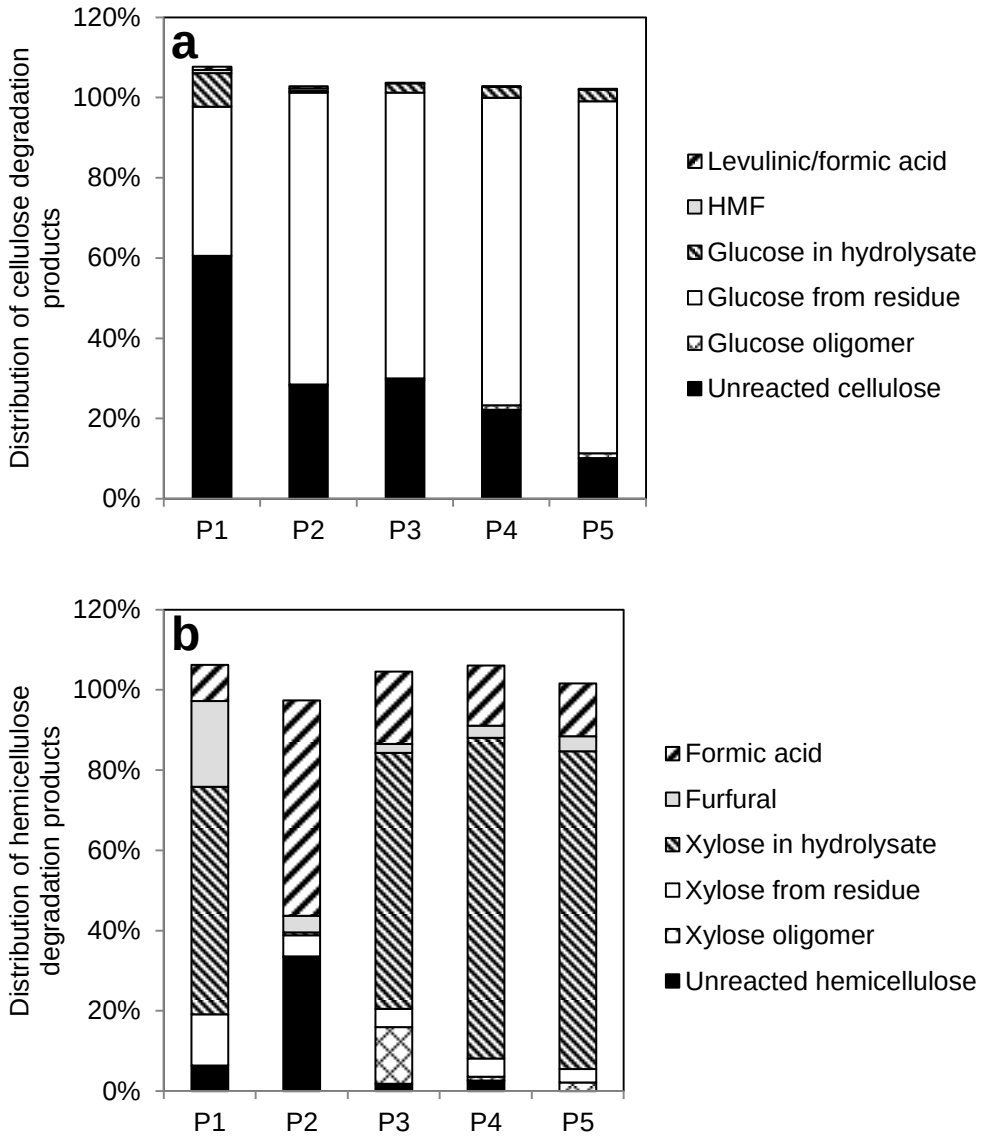


Figure 2.3. Sugar degradation products from cellulose (a) and hemicellulose (b) resulting from pretreatment schemes described in Table 2.3.

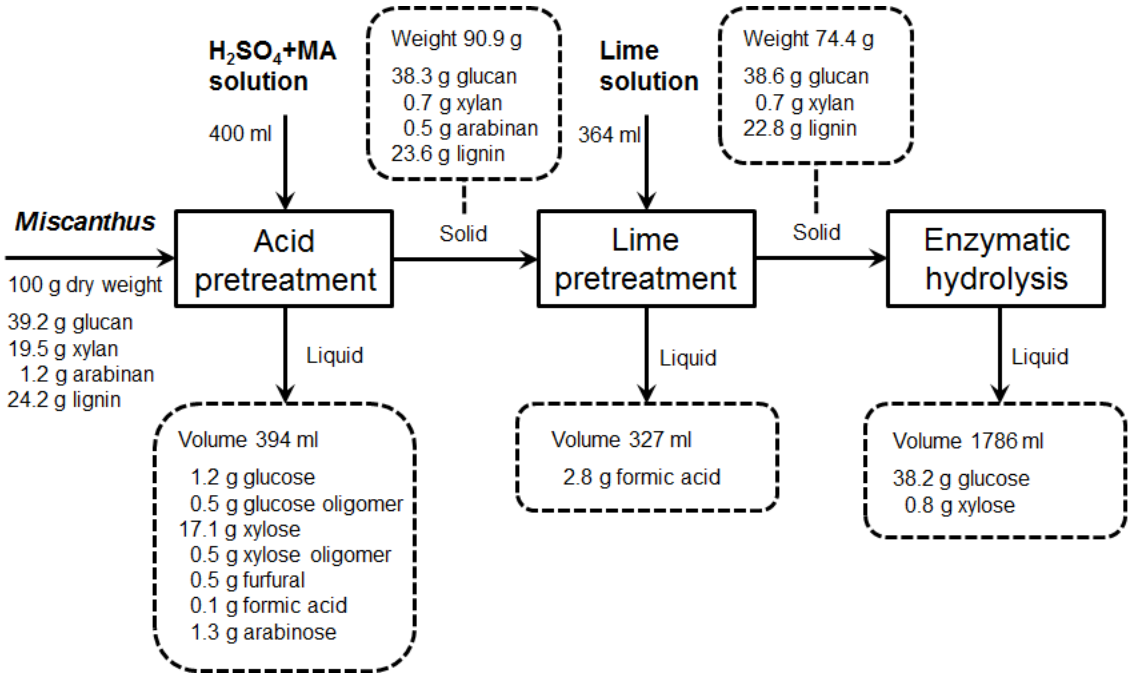


Figure 2.4. Material balance flow diagram of two-stage pretreatment process with combined H_2SO_4 and maleic acid in the acid stage.

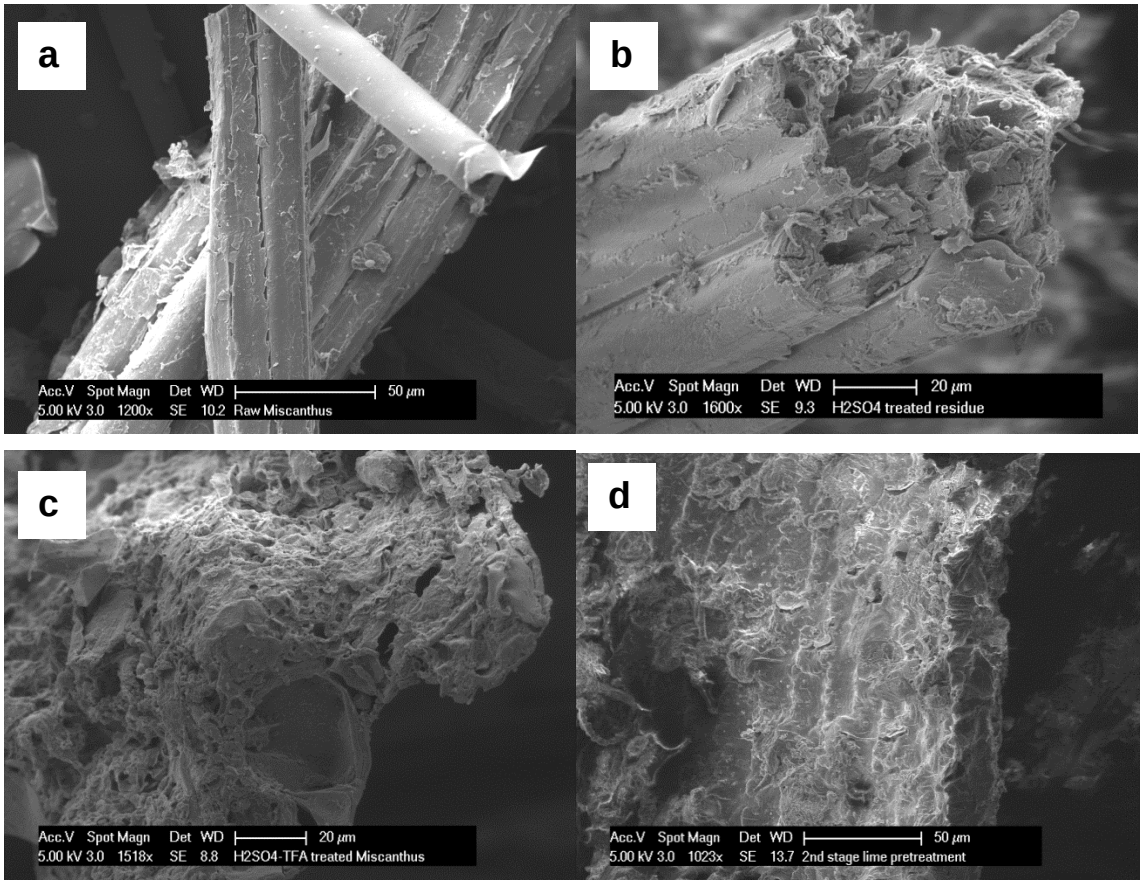


Figure 2.5. ESEM images of treated *Miscanthus* under various stages (Magnification: 1023×-1600×). (a) Raw *Miscanthus*; (b) After 1st stage sulfuric acid pretreatment; (c) After 1st stage combined acid pretreatment; (d) After 2nd stage lime pretreatment.

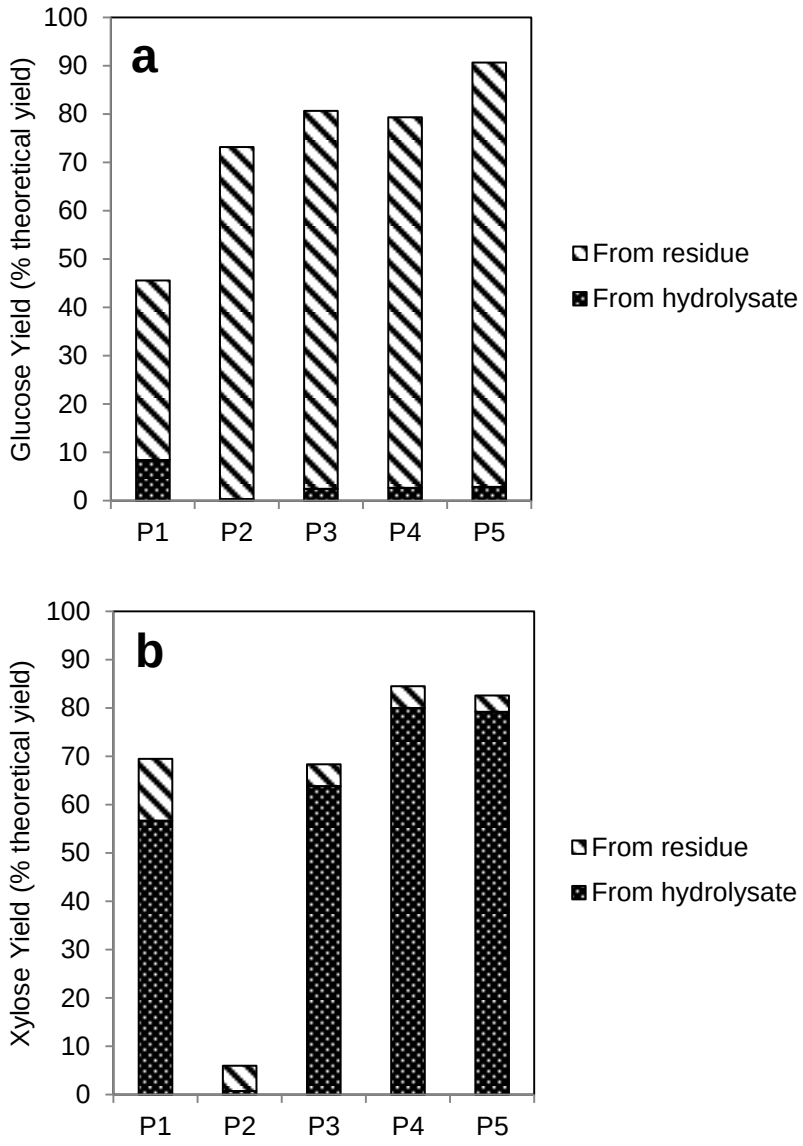


Figure 2.6. Glucose (a) and xylose (b) yields under pretreatment schemes described in Table 2.3.

Table 2.1. Coded values of the tested variables at various levels

	Variables	**Range and levels**				
		-2	**-1**	**0**	**1**	**2**
Acid stage	Acid dosage (wt%)	0.25	0.50	0.75	1.00	1.25
	Temperature (ºC)	130	145	160	175	190
	Residence time (min)	5	15	25	35	45
	Variables	**Range and levels**				
		-1.414	**-1**	**0**	**1**	**1.414**
Alkaline stage	Lime loading (g $Ca(OH)_2$/g biomass)	0	0.0117	0.04	0.0683	0.08
	Temperature (ºC)	175	185	210	235	245

Table 2.2. Yields of primary phenols (concentration > 5 mg/L) in the hydrolysates (in mg/L)

Conditions	Acid stage			Alkaline stage				
	Medium (0,0,0)	**Harsh (1,1,1)**	**Mild (-1,-1,-1)**	**Medium (0,0)**	**Higher lime loading (1.4,0)**	**Lower lime loading (-1.4,0)**	**Higher temp. (0,1.4)**	**Lower temp. (0,-1.4)**
P-hydroxybenzaldehyde	5.4	8.6	6.4	4.9	3.1	19.1	1.2	15.4
P-hydroxybenzoic acid	6.3	15.0	4.3					
Vanillin	30.9	97.7	20.2	10.8	4.7	41.7	2.7	17.6
Vanillic acid	16.6	63.0	8.6	1.4	1.6	14.4		7.0
Syringaldehyde	10.3	5.7	10.8	4.0		26.2		7.6
Syringic acid	8.2	27.5	4.0	1.1	1.2	9.0		4.1
P-coumaric acid	80.9	17.9	131.9	3.4	4.6	9.4		20.6
Ferulic acid	44.8	5.8	33.6					
Syringol				16.1	22.4	9.9	30.2	4.2
Methylhydroquinone				1.4		0.6	17.9	0.2
2-(4-hydroxyphenyl) propionic acid				4.9	9.2	1.9	2.1	2.8
3-(4-hydroxyphenyl) propionic acid				1.7	2.3	1.0	9.2	0.2
3-vanillyl propanol				2.6	2.6	7.0	3.7	1.6
3-hydroxybenzoic acid				2.1	2.6	4.2	5.7	3.1

Note: Concentrations of syringol down to 3-hydroxybenzoic acid were shown in ratios to 1.67 mg/L phthalic acid. 1.67 mg/L phthalic acid equals 1.5-4.0 mg/L phenols depending on the phenols type.

Table 2.3. Operational conditions of various pretreatment schemes for comparison

Pretreatment schemes	Acidic stage	Alkaline stage
P1	1.0 wt% H_2SO_4, 170 °C, 15 min	
P2	0.024 g/g $Ca(OH)_2$, 202 °C, 30 min	
P3	0.73 wt% H_2SO_4, 150 °C, 6 min	0.024 g/g $Ca(OH)_2$, 202 °C, 30 min
P4	0.375 wt% H_2SO_4 + 4 mg/L TFA, 150 °C, 6 min	0.024 g/g $Ca(OH)_2$, 202 °C, 30 min
P5	0.548 wt% H_2SO_4 + 15.6 g/L MA, 150 °C, 6 min	0.024 g/g $Ca(OH)_2$, 202 °C, 30 min

Table 2.4. Major products in the prehydrolysates under various pretreatment schemes

Pretreatment Schemes		**P1**	**P2**	**P3**	**P4**	**P5**
Weak acids (g/L)	Acetic acid	8.5	11.2	5.7 (81%)	7.3 (80%)	8.6 (79%)
	Formic acid	1.8	9.4	1.3 (23%)	1.2 (20%)	1.1 (25%)
	Levulinic acid	0.6	0.4	0.1	0.1	0.2
Furans (g/L)	Furfural	7.7	1.5	0.9 (97%)	1.1 (96%)	1.5 (88%)
	HMF	0.5	0.4	0.1	0.1	0.1
Phenols (mg/L)	Total phenols (g/L)	4.1	5.6	3.0 (29%)	3.9 (18%)	2.9 (24%)
	P-hydroxybenzaldehyde	18.8	9.2	9.7	3.3	2.3
	P-hydroxybenzoic acid	13.7	5.3	2.8	1.1	0.9
	Vanillin	76.0	7.4	21.0	7.3	6.4
	Vanillic acid	37.4	4.9	4.6	1.8	1.8
	Syringaldehyde	34.3	3.5	13.2	5.6	5.1
	Syringic acid	17.1	3.0	2.5	1.2	0.9
	P-coumaric acid	102.0	3.2	66.7	38.0	27.2
	Ferulic acid	46.1	5.3	11.0	5.0	4.1
	Syringol		9.1	0.7	1.2	0.4
	3-vanillyl propanol		3.2	0.4	0.5	0.1
	Hydrocinnamic acid		48.6	0.7	0.2	0.2
	Homovanillic acid		6.6	0.3	0.4	0.1

Note: (1) Percentage of products derived from the hydrolysates in alkaline stage was shown in the brackets; (2) Concentrations of syringol down to homovanillic acid were shown in ratios to 1.67 mg/L phthalic acid. 1. 1.67 mg/L phthalic acid equals 1.5-4.0 mg/L phenols depending on the phenols type.

Table 2.5. Concentrations of major compounds in the SSCF hydrolysates under various pretreatment schemes (in g/L)

Pretreatment schemes	**P1**	**P2**	**P3**	**P4**	**P5**
Glucose at 0 h	5.3	1.3	1.8	2.2	2.3
Glucose at 48 h	18.0	22.3	0.0	0.0	21.5
Glucose at 96 h	18.6	21.8	0.0	0.0	0.1
Xylose at 0 h	14.0	0.4	13.1	18.1	17.8
Xylose at 48 h	14.3	1.8	6.0	12.3	18.4
Xylose at 96 h	13.9	1.9	3.1	8.8	12.1
Ethanol at 48 h	0.2	1.0	11.0	12.9	1.6
Ethanol at 96 h	1.4	3.1	11.1	14.7	15.9
Ethanol yield (g/g of dry *Miscanthus*)	0.011	0.026	0.093	0.132	0.145
Ethanol yield of theoretical maximum	10%	20%	57%	63%	62%

CHAPTER 3
COMBINED BIOMIMETIC AND INORGANIC ACIDS HYDROLYSIS OF HEMICELLULOSE[2]

Abstract

Combined acid catalysis was employed as a pretreatment alternative with combined acid catalysts blending sulfuric acid with two biomimetic acids, trifluoroacetic acid (TFA) and maleic acid (MA), respectively. The influences of acid blending ratio, temperature, and acid dosage on pretreatment performance were investigated. A synergistic effect on hemicellulose decomposition was observed in the combined acid hydrolysis, which greatly increased xylose yield, although TFA/MA would induce more total phenols. Besides, combined TFA pretreatment could efficiently prevent xylose degradation. Fermentation tests of the acid-catalyzed hydrolysates with overliming showed that compared to H_2SO_4 pretreatment, TFA and MA pretreatments improved overall ethanol yield with an increase by 27-54%. Combined acid catalysis was shown as a feasible pretreatment method for its improved sugar yield, reduced phenols production and catalyst costs.

Keywords

Combined acid hydrolysis, biomimetic approach, *Miscanthus*, hemicellulose decomposition, xylose degradation

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3.1 Introduction

As one promising alternative energy source, lignocellulose-derived fuels and chemicals have been drawing great attention for development in the recent years to supplement conventional fossil fuels (Somerville et al., 2010). For bioconversion of lignocellulose, the remarkable technical (Jacobsen and Wyman, 2000) and economic challenges (Eggeman and Elander, 2005) imposing on the entire process along with intensive energy demand (Wyman et al., 2005) put pretreatment as the linchpin of the biofuel technologies.

Over the past decades, a wide range of pretreatment technologies have been developed and many leading pretreatment methods could achieve efficient delignification and high hexose yield from cellulose (Kumar et al., 2009; da Costa Sousa et al., 2009). However, optimizing overall fuel production will, in addition to high hexose yields, require efficient co-utilization of pentose from hemicellulose in biomass (Lin and Tanaka, 2006). For hemicellulose hydrolysis, dilute sulfuric acid is the most cost effective pretreatment methods for pentose recovery among others (Saha, 2003). However, it would unavoidably further degrade a fair amount of pentoses into fermentation inhibitory compounds such as furans and their derivatives. Contrarily, another efficient group of catalysts, hemicellulase, catalyses hemicellulose only to monosaccharides without over-degradation, but its prohibitive production cost would price it out of the market (Collins, Gerday and Feller, 2005).

To incorporate the individual hydrolysis advantages of sulfuric acid and hemicellulolytic enzyme and avoid their drawbacks, a biomimetic approach emerged recently with a type of newly designed hybrid catalysts (Lu and Mosier, 2007). The biomimetic catalysts were aimed to possess the superior selectivity of hemicellulase for hemicellulose decomposition over sugar degradation by structurally mimicking catalytic functional groups in the enzymes. It was observed that the key function domain is composed of a pair of carboxylic amino acid residues serving as proton donor and acceptor, respectively. The two-amino acid system fuels hydrolysis of glycosidic bonds in the sugar chain through protons transfer between two acid residues (Mosier et al., 1999). Accordingly, dicarboxylic acids, analogous to catalytic structures, were proposed as biomimetic catalysts and have shown significantly increased sugar yields compared to

sulfuric acid while keeping relatively low catalyst cost. Among the tested dicarboxylic acids, maleic acid (MA) showed the most favorable catalysis selectivity (Mosier et al., 2001). Apart from dicarboxylic acid, trifluoroacetic acid (TFA) was investigated independently as a substitute of mineral acids for biomass pretreatment, and reported to have similar selectivity of sugar polymer hydrolysis (Marzialetti et al., 2008; Dong et al., 2009). A strong monocarboxylic acid, TFA has a low boiling point and was thereby suggested for reuse by evaporation in pretreatment (Albersheim et al., 1967).

Although showing great promise in high sugar recovery rate and cost reduction, MA as well as TFA is still too expensive to be economically applicable compared with sulfuric acid. Moreover, new potential challenges are present, such as intensive energy input of TFA reuse by evaporation, and unknown toxicities of these organic acids to fermentation microbes.

To address the above issues, in this study, the concept of combined organic and inorganic acids in asymmetric synthesis (Yamamoto and Futatsugi, 2005) was adopted to develop improved biomimetic acid catalysts. Over the course of synthesis process, combined acid system would result in both higher reactivity and selectivity than individual catalysts, because adding a second catalyst that slows down the reaction could suppress unwanted side reactions (Schreiner, 2010). In this work, the combined acid catalysts were composed of sulfuric acid and a biomimetic acid (MA or TFA). It was hypothesized that the catalysts would integrate the cost advantage of sulfuric acid and selectivity advantage of biomimetic acids, and would achieve similarly high reactivity and selectivity to sulfuric acid and biomimetic acids individually. Through blending with two types of acids, the catalysts would also allow to reduce the impact of biomimetic catalysts by trimming their dosage. Here we applied two combined acid catalysts for hemicellulose hydrolysis, with *Miscanthus* as model feedstock at a high solids loading. Hydrolysis performance was evaluated in terms of sugars recovery and inhibitory by-product yields. The influences of three major factors, acid blending ratio, operating temperature and acid dosage, were systematically studied. Furthermore, fermentability of hydrolysates was examined using engineered *Saccharomyces cerevisiae* to compare the effectiveness of various catalysis systems. The objective of this study was to evaluate the feasibility of combined acid catalysts in hemicellulose hydrolysis and preliminarily

characterize the specific function of the catalysts throughout the hydrolysis process.

3.2 Materials and Methods

3.2.1 Raw Material and Characterization

Miscanthus x giganteus used in this study was harvested and collected in spring 2008 from Energy Biosciences Institute (EBI) Energy Farm in Urbana, IL. The raw material was air dried at temperature below 45 ºC with final dry matter content of 91-94%. The dried *Miscanthus* was milled using a hammer mill (Sears Roebuck and Co.) with a 1/4-in. (6.35 mm) sieve, homogenized in a single bucket and then stored under dry conditions at room temperature for experiments.

Composition analysis was conducted following the National Renewable Energy Laboratory (NREL) standard procedures (Technical Report NREL/TP-510-42618) to determine the major sugars contents. The sugar composition of the raw biomass was as follows: glucan 39.6±1.6%, xylan 18.7±0.9%, arabinan 3.5±0.3%, and mannan 1.5±0.6%.

3.2.2 Dilute Acid Pretreatment

Pretreatment was conducted in a floor-stand stirred-tank batch reactor (Model 4572, PARR Instrument Co., Moline, IL). The reactor was made of T316 stainless steel with extreme operation conditions of 34.5MPa and 375 ºC, and equipped with a 1.8 L pressure vessel. A 400 W heating mantle was mounted to heat the reactor and a temperature controller (Model 4842, PARR Instrument Co., Moline, IL) was affixed. Control of operating pressure was indirectly achieved through temperature control. The total loaded reactants were 600 g with a solid loading of 20 wt% dry-based *Miscanthus* samples. Prior to the pretreatment, *Miscanthus* was impregnated with acid solutions to be used for the catalysis for 9 h at ambient temperature. After loading biomass along with catalysts, the vessel was clamped shut and nitrogen was introduced to fill the headspace with constant pressure of 95 psi. The vessel was then heated at 6-8 ºC/min rate and controlled at a constant temperature once it reached the pre-set value, with agitation at 400 rpm. When the pretreatment was complete, the vessel was cooled down to 60 ºC in approximately 10 min and the pressure was then released immediately.

In our previous work, dilute sulfuric acid pretreatment of *Miscanthus* was

determined using central composite design (CCD), a standard Response Surface Methodology (RSM), with three important variables: H_2SO_4 dosage (pH of catalytic solutions), temperature, and retention time. For the conditions studied, optimal conditions for hemicellulose hydrolysis were achieved at 0.73 wt% sulfuric acid (pH=1.07), 150 ºC, and 6.1 min, with 20 wt% solid loading rate (data not published). The optimal conditions here were defined to be able to obtain the highest xylose yield as well as lowest production of sugar degradation products. In this work of combined acid pretreatment, two combined acid systems, H_2SO_4 blended with TFA and MA, were looked into individually under the above conditions. Besides, pretreatments with two acid systems were carried out at two other temperatures (130 ºC, 170 ºC) and pH values (0.87 and 1.35) as well, respectively. For each acid system under varied operating conditions, five acid blending ratios were applied with H_2SO_4 fraction at 0% (pure TFA or MA), 25%, 50%, 75%, and 100% (pure H_2SO_4). In the acid-catalyzed hydrolysis of cellulose and hemicellulose, specific catalysis is generally used to explain the mechanism (Sidiras and Koukios, 1989; Malester, Green and Shelef, 1992), which means the reaction rate is proportional to the proton concentration and independent of other species capable of donating protons (Lewis acids). In this regard, same pH value was ensured to provide same amount of protons in the reaction under various acid blending ratios.

In addition, the individual effect of each acid component was identified under optimal conditions but with various acid blending ratios. The combined acid system under certain acid blending ratio was disintegrated into two parts, each with one type of acid at the same applied concentration as in the combined system, and the rest was filled with water to replace the other missing acid part.

After the pretreatment, the treated wet biomass was filtered through Whatman No.1 fitler paper in a Buchner funnel. Hydrolysates (liquid fraction) were analyzed for sugar oligomers and monomers, sugar degradation products as well as phenolic compounds, and were also used as substrates in the following fermentation tests. On the other hand, solid residues were analyzed for xylose and glucose contents to calculate the mass balance throughout the pretreatment process.

3.2.3 Enzymatic Hydrolysis

Enzymatic hydrolysis of pretreated biomass was conducted based on NREL standard procedure (Technical Report NREL/TP-510-42629). Treated *Miscanthus* samples after filtration were hydrolyzed in 50 mM sodium citrate buffer (pH 4.8) at a loading of 2.0 wt% dry matter content. Total volume in each flask was 50 ml. Sodium azide was added to 0.2 mg/ml to prevent microbial contamination during the digestion. Applied enzyme loadings were 60 FPU/g biomass of cellulase (Spezyme CP, Genencor), 2 CBU/FPU of β–glucosidase (Novozym 188, Sigma-Aldrich) supplemented with xylase (Multifect Xylanase, Genencor). Hydrolysis of each sample without enzyme addition was taken as control test. The test flasks were incubated at 50 ºC and shaken at 150rpm. Samples were taken at 24, 48, 72 h and then boiled for 5 min followed by immediately chilling in ice bath to degenerate the enzyme. Hydrolysates were filtered and the supernatant was stored at -20 ºC for future sugar analysis.

3.2.4 Fermentation Experiments

An efficient xylose-fermenting strain (DA24-16) was used for fermentation experiments using pretreated hydrolysates. The DA24-16 was constructed using *S. cerevisiae* D452-2 (*MATalpha, leu2, his3, ura3,* and *can1*) through the artificial isozyme system and laboratory evolutionary approach (Ha et al., 2011). Methods for strain cultivation and fermentation experiments described in Ha, et al. (2011) were followed. Yeast strains were routinely cultivated at 30 ºC in YP medium (10 g/L yeast extract, 20 g/L Bacto peptone) containing 20 g/L glucose. Cells at mid-exponential phase were harvested and inoculated after washing twice by sterilized water. Hydrolysates after combined acid pretreatment were adjusted to pH at 5.0±0.1 in two ways: one through direct addition of H_2SO_4, and the other by overliming (addition of $Ca(OH)_2$ to pH 10-11 first, followed by H_2SO_4 down to pH 5). Thereafter, pH adjusted hydrolysates were fermented by DA 24-16 at 30 ºC for 52 h with initial OD_{600} of ~10 under oxygen limited conditions.

The individually inhibitory effects of TFA and MA on cell growth and ethanol fermentation were also investigated. Solutions with 2 g/L glucose and 25 g/L xylose were prepared with various concentrations of TFA (2, 4, 8, 16 ml/L) and maleic acid (10.0, 22.0, 61.5, 140.0 g/L) at pH 5.0, respectively. The concentrations of the above sugars and

acids were determined to be close to those in dilute acid pretreatment tests. Sugar solutions at same initial concentration without TFA or MA were used as control. Yeast strain was inoculated with initial OD_{600} of around 5, and then incubated at 30 ºC for 98 h. During all fermentation tests, samples were taken periodically to measure OD, ethanol, sugars, glycerol, xylitol, and acetate concentrations.

3.2.5 Analytical Methods

NREL standard protocol (Technical Report NREL/TP-510-42621) was followed for dry matter contents determination. Wet *Miscanthus* samples were analyzed after drying at 105 ºC for 4 h and then desiccating for 1 h. Hydrolysates were hydrolyzed by 4% w/w sulfuric acid at 121 ºC for 60 min to break down the remaining oligosaccharides in the solutions to monosaccharides.

For pretreatment tests, HPLC system (Shimadzu) equipped with a refractive index detector (Waters) was used for quantitative analysis of sugars, furans, weak acids and ethanol. Shodex sugar SP0810 column with ionic form H^+/CO_3^- deashing guard column was used to measure major sugars including cellobiose, glucose, xylose, galactose, arabinose and mannose. Nanopure water was used as mobile phase with a flow rate of 0.6 ml/min at 85 ºC. Sugar degradation products (Acetic acid, furfural and hydroxymethylfurfural (HMF)) and furan degradation products (formic acid and levulinic acid) were analyzed by Bio-Rad Aminex HPX-87H column with correspondent guard column with 5mM sulfuric acid as mobile phase at 0.6 ml/min. Higher column temperature at 65 ºC was applied to analyze acetic acid, furfural and HMF while lower temperature at 40 ºC for formic acid and levulinic acid.

Hydrolysate samples were extracted with ether twice at 3:1. The ether phase was then concentrated by nitrogen bubbling. The concentrated ether samples were analyzed for phenolic compounds by GC/MS system consisted of an Agilent 7890A gas chromatograph, an Agilent 5975 mass selective detector and Agilent 7683B (Agilent Inc, Palo Alto, CA) autosampler. Injections were performed on a 60 m HP-5MS column with 0.25 mm inner diameter and 0.25 μm film thickness (Agilent Inc, Palo Alto, CA) with an injection port temperature of 250 ºC, the interface set to 250 ºC, and the ion source adjusted to 230 ºC. The helium carrier gas was set at a constant flow rate of 1.5 ml/min.

The temperature program was 5-min isothermal heating at 70 ºC, followed by an oven temperature increase of 5 ºC/min to 300 ºC in 5 min. The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy in m/z 50-800 scan range. The chromatograms and mass spectra were evaluated using MSD ChemStation (Agilent, Palo Alto, CA) and AMDIS (NIST, Gaithersburg, MD) programs. The spectra of all chromatogram peaks were compared with electron impact mass spectrum libraries NIST08 (NIST, MD), WILEY08 (Palisade Corporation, NY) and a custom library built with authentic standards. Hydrolysate samples were also analyzed for estimation of total phenols content colorimetrically by the Folin-Ciocalteu method (Scalbert, Monties and Janin, 1989). Samples were diluted 40-100 times by water to obtain a final absorbance in 0.1-0.5. Calibration was conducted with gallic acid and total phenols were expressed in gallic acid equivalent.

For fermentation tests, cell growth was monitored by optical density (OD) at 600 nm using UV-visible Spectrophotometer (Biomate 5, Thermo, NY). Glucose, xylose, xylitol, glycerol, acetate, and ethanol concentrations were determined by HPLC system (Agilent Technologies 1200 Series) equipped with a refractive index detector using a REzex ROA-Organic Acid H^+ (8%) column (Phenomenex Inc., Torrance, CA). The column was eluted with 5mM sulfuric acid at 0.6 ml/min at 50 ºC.

All the chemicals used in this work were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (St. Louis, MO).

3.2.6 Statistical Analysis

Untreated *Miscanthus* was analyzed in sextuple. Most pretreatments were conducted in duplicate and triplicate, and relative standard deviations were within 5% in all cases. Enzymatic hydrolysis and fermentation experiments were performed in triplicate and duplicate individually, and a 95% confidence level was applied for data analysis.

3.3 Results and Discussion

3.3.1 Performance of Hemicellulose Hydrolysis

The process of hemicellulose hydrolysis was evaluated in terms of reactivity and selectivity of related reactions individually in this work.

(1) Reactivity of combined acid catalysts

Under optimal conditions, xylose yields from hemicellulose decomposition by two studied combined acid catalysts were shown in Figure 3.1. To bring pH to 1.07, the applied concentrations in a single acid system are 0.075 mol/L for H_2SO_4 (eq. 0.73 wt%), 0.104 mol/L for TFA, and 0.530 mol/L for MA individually.

In Figure 3.1(a), we observed that compared with single H_2SO_4 pretreatment, single TFA pretreatment increased xylose yield by 38%, from 10.7% up to 14.8% dry biomass. This initially implied that at the same pH level, the anion part of TFA provided additional stimulating effect on hemicellulose decomposition besides the proton function over the hydrolysis process dissociated from TFA. Furthermore, along with the increase of H_2SO_4 ratio in the combined acid system, xylose yield was only slightly influenced by low additions of H_2SO_4, and then dropped significantly when H_2SO_4 accounted for more than half of the catalysts. If two acid components provided independent and proportional effects to their concentrations, the expected xylose yield trend along various acid blending ratios would be a straight line from 14.8% down to 10.7%, based on the principles of linear interpolation. In contrast, the unexpected S-shaped curve shown in Figure 3.1(a) indicated an interaction effect existing between the two acid components, which drove the actual xylose yield away from the projected linear curve by a great margin. This effect brought about a series of hydrolysis results with xylose yields highly reliant on the acid blending ratios. To further explore this interaction effect, the combined acid system under a specific acid blending ratio was disintegrated into two parts, each with only one acid component at the same applied concentration as in the combined system. This generated two similar curves of monotone increasing function along with the increase of acid usage, across with each other as shown in Figure 3.1(a). These two curves presented individual effect on hemicellulose decomposition by H_2SO_4 and TFA, respectively. Subsequently, the independent sum of these two curves generated a hypothetical curve as shown in dashed line, which clearly excluded the interaction effect between the two acid components. In this case, the real xylose yield curve by combined acid catalysis was always above the hypothetical curve, which again verified the interaction effect and further showed an evident positive synergistic effect between proton and TFA anion. The strongest synergistic effect was achieved at 50:50 acid

blending ratio when the widest gap of xylose yield (4.2% dry biomass) between two curves was detected.

Hydrolysis results by H_2SO_4-MA system showed in Figure 3.1(b) depicted a different profile compared to H_2SO_4-TFA system. First, MA itself showed stronger hydrolysis capability than either H_2SO_4 or TFA at low applied levels. At only 25% of the maximal applied concentration, MA could achieve roughly 80% of the maximal xylose yield. However, xylose yield tended to level off with increasing applied dosage at around 13.5%. It seemed there was a limit on the total amount of hemicellulose capable of being hydrolyzed, and in this case, the limit was 63.5%. In general, hemicellulose can be divided into an easy-to-hydrolyze portion and a hard-to-hydrolyze part, and the latter accounts for 35% typically for various types of materials (Jacobsen and Wyman, 2000). Combined the above information, it was probably that MA could mainly hydrolyze the easy-to-hydrolyze hemicellulose in *Miscanthus*. In addition, these relatively unchanged xylose yields raised the hypothetic curve way above the real curve. Figure 3.1(b) also showed that combined MA pretreatment profile was consistent with that of combined TFA pretreatment discussed above, indicating a synergistic effect between H_2SO_4 and MA, since the real curve by combined acid catalysis was flat instead of incliningly straight. This generally flat pattern was possibly attributed to the limited capability of MA for hemicellulose hydrolysis as pointed out above.

(2) Selectivity of combined acid catalysts

Hemicellulose hydrolysis is in fact a continuous depolymerization process, associated with a string of consecutive reactions producing gradually molecular weight-decreasing intermediates, which include oligomeric and monomeric xylose, furfural, and the end product formic acid.(Lee, Iyer and Torget, 1999).To better understand the catalytic mechanism of combined acid system, distributions of various hemicellulose degradation products after hydrolysis under different acid blending ratios were quantitatively identified as shown in Figure 3.2. In addition, the term “selectivity” was used here to describe the catalysis favorability between hemicellulose decomposition and xylose degradation. For consecutive reactions A→B→C, selectivity is defined as $S_B=n_B/n_A$, where n_B refers to the amount of B generated from A, and n_A refers to the amount of A consumed in total. Selectivity of each reaction throughout hemicellulose

hydrolysis as well as hemicellulose degradation rate was compared in Figure 3.3.

As we can see in Figure 3.2(a), compared with single H_2SO_4 pretreatment, single TFA pretreatment led to significant higher xylose yield, obviously through more efficient decomposition of hemicellulose. On the contrary, change of furfural production was not detected. For combined acid catalysis, along with increase of H_2SO_4 ratio, hemicellulose was decomposed to a less extent, and thereby less xylose was generated. This implied that TFA is in favor of hemicellulose decomposition. A clearer view of the catalytic mechanism of combined TFA hydrolysis can be obtained in Figure 3.3(a) through the selectivity profiles. Except for selectivity of furfural, all the other parameters presented strong correlation with acid blending ratio. The more TFA applied in the solution, the higher value of hemicellulose decomposition rate as well as selectivity of xylose, while generally the lower selectivity of xylose oligomer. These combined information again verified TFA would facilitate decomposition of both hemicellulose and oligomeric intermediates, and indicated TFA's favorable prevention of xylose degradation, although to much less extent as shown by the changing magnitude in Figure 3.3(a). The observation was in accordance with other reports that dilute TFA hydrolysis resulted in higher amount of soluble sugars and less sugar degradation (Marzialetti et al., 2008; Dong et al., 2009). Moreover, most effects on the reaction favorability occurred in the range of H_2SO_4 ratio from 50% to 100%. This implied that only half of the applied TFA concentration would be enough to bring the significant effect on the hydrolysis.

TFA has been found to be able to dissolve cellulose efficiently. It involves the formation of an intermediate through esterification with hydroxyl group in cellulose (Geddes, 1956). Since cellulose and hemicellulose share similar structures, the mechanism of esterification could be adopted for hemicellulose decomposition by TFA, to explain its enhanced hydrolysis efficiency. In contrast, proton would attack and hydrolyze the ether linkages between sugar polymers (Nimlos et al., 2006). Therefore, in the combined acid system, we could imagine that both H_2SO_4 and TFA offered their unique functions for efficient hemicellulose decomposition. However, in the subsequent xylose degradation process, their effects are completely different as observed above. In xylose degradation, the rate-limiting step is the initial reaction with protonation of 2-OH on the sugar ring to form 2,5-anhydride intermediate (furfural precursor) (Qian et al.,

2005a). It has been found that certain chemicals, like ethanol, could substantially increase glucose yield in dilute acid hydrolysis of cellulose. One possible mechanism is related to strong interaction of ethanol with glucose which subsequently shields glucose from protonation (Qian et al., 2005b). Analogously, in this case, TFA anion might inhibit xylose degradation through disturbing the protonation step. The other possible explanation is that TFA could influence water structure in the solution and induce formation of hydrogen bond between water molecules and hydroxyl groups on the sugar rings. Water molecules bonded to sugar hydroxyls could easily terminate the reaction to furfural by extracting/donating a proton from/to the intermediate (Qian et al., 2005a). It is important to note that a certain amount of TFA could achieve required effect, with additional usage leading to only marginal influence. The pretreatment results showed that the optimal pretreatment performance was achieved under 50:50 acid blending ratio, with nearly same yield of xylose and furfural compared to single TFA pretreatment, but only a half of the catalyst costs.

The performances for combined MA-H_2SO_4 pretreatment are different from those for combined TFA-H_2SO_4 pretreatment. In Figure 3.2(b) and Figure 3.3(b), it appeared that MA could facilitate hemicellulose decomposition, but not apparently prevent xylose degradation as TFA did. As MA ratio was raised in combined acid catalysts, fraction of hemicellulose as well as xylose oligomer was significantly reduced, while fraction of furfural did not change perceptibly. For single H_2SO_4 pretreatment, hemicellulose decomposition rate was remarkably lower and selectivity of xylose oligomer was higher compared to combined MA pretreatment. Meanwhile, selectivity of xylose did not have detectible correlation with the type of applied acid catalysts. These results are different from what other limited research found. Lu and Mosier (2008) and Kootstra et al. (2009) reported to have much less furfural formation during MA pretreatment compared to H_2SO_4 pretreatment for corn stover and wheat straw, respectively. However, there might not be a contradiction between these two opposite observations. First, solid loading in their research was lower than 10 wt%, which would have sizably reduced furfural production. Secondly, for various acid catalysts comparison, they kept acid concentration the same, while in this study pH value was instead kept the same, which would increase the usage of MA and thereby induce more furfural produced.

Since MA also belongs to carboxylic acid as TFA, it is easy to understand that stimulation of hemicellulose decomposition by MA might be attributed to its esterification with hydroxyl groups in hemicellulose. For xylose degradation, further work is needed to characterize the different performances between MA and TFA. This might be due to the fact of MA's different structure as monocarboxylic acid or its low pK_a value that gave it differently functionality throughout the hydrolysis. Similarly as TFA, it is important to point out that only a small amount of MA would result in significant improvement of hemicellulose hydrolysis. Most changes of hemicellulose related products distribution were achieved in the first 25% addition of MA, and any further addition of the acid would not have detective effect. Therefore, compared to single MA pretreatment, 75:25 H_2SO_4-MA led to similar xylose yield and furfural production, but with 75% less catalyst costs.

Some other related studies of hemicellulose hydrolysis by dilute acids included a work on corn stover by sulfuric acid hydrolysis at relatively low temperature of around 100ºC (Jin et al., 2011). Throughout the pretreatments, over 90% of xylose yield was achieved and less than 5.5% turned to furfural. However, the pretreatments were carried out with a low solid loading (4.8%), which could easily hydrolyze hemicellulose and favor the xylose accumulation. In other dilute acid pretreatment works with higher solid loading of 10-15%, xylose yield was significantly lower (67-72%) while furfural formation was in 1-2% range (Lee et al., 2011; Kim et al., 2011). In this study, with 20% solid loading under the optimal conditions, 66% and 9% of hemicellulose converted to xylose and furfural, respectively, by single H_2SO_4 pretreatment. By combined acid pretreatment (either TFA or MA), up to 81% of xylose yield could be reached.

3.3.2 Effects of Temperature and Acid Dosage

In addition to the optimal conditions, combined acid pretreatments were also carried out at two other temperatures (130 ºC and 170 ºC) and acid dosages (pH 0.90 and 1.40), to investigate how temperature and acid dosage affect hemicellulose hydrolysis. Xylose yields and furfural productions under various operating conditions were presented in Figure 3.4 and Table 3.1, respectively.

For combined TFA pretreatment, when raising the temperature or acid dosage from

the optimal point, xylose yield did not increase but furfural formation built up significantly. In contrast, xylose yield went down significantly with either temperature of acid dosage from the optimal point down to the lowest level. This indicated that temperature of 150 ºC and pH of 1.07 would be necessary and enough to overcome the activation energy barrier of hemicellulose decomposition, and any harsher conditions would be redundant and easily induce furfural formation. Besides, all operating conditions presented S- or reverse U-shaped curves under various acid blending ratios, which indicated strong synergistic effect existing in all cases. The optimal conditions derived from dilute H_2SO_4 pretreatment optimization also appeared to be optimal for single TFA pretreatment and combined TFA pretreatment. Interestingly, under all extended conditions other than the optimal case, combined TFA pretreatment induced 30% less furfural production than single H_2SO_4 pretreatment, which evidently verified that TFA could strongly inhibit xylose degradation under given operating conditions and combined acid system would intensify this effect via synergistic interaction.

For H_2SO_4-MA system, since single MA pretreatment would result in similar xylose yield as single H_2SO_4 pretreatment, synergistic effect was hardly observed. Beside, not much change of xylose yield was detected among various conditions. It appeared that compared to combined TFA pretreatment, temperature and acid dosage have less influences on hemicellulose hydrolysis in combined MA pretreatment. However, the optimal conditions adopted here showed to be also optimal for combined MA pretreatment, since highest xylose yield along with lower furfural production could be achieved. In addition, unlike the case of TFA, combined MA pretreatment always showed induced furfural formation compared to single H_2SO_4 pretreatment because MA in the system played a key role in helping xylose degradation.

3.3.3 Cellulose Hydrolysis

Similar as hemicellulose, during the dilute acid pretreatment, cellulose was also converted into various degradation products. The yields of these cellulose related products after hydrolysis were listed in Table 3.2. In this study, the pretreatment conditions were designed with the main aim of hemicellulose hydrolysis, which was inevitably accompanied by low glucose yields. For the acid hydrolysis tests, almost more

than 90% of cellulose stayed in the biomass, although with 54-75% of it digestible by cellulolytic enzymes. At such low level of glucose yields, no evident effect of acid blending ratio was found on either glucose formation or cellulose digestibility.

3.3.4 Phenolic Compounds Production

Throughout pretreatment process of biomass, phenolic compounds are inevitably generated along with lignocellulose decomposition and breakdown of lignin. As strong fermentation inhibitory compounds, these lignin derivatives were intensively studied and characterized during fractionation processes of some important herbaceous crops such as corn stover, wheat straw, rice straw and flax straw (Buranov and Mazza, 2008). However, production of phenolic compounds after pretreatment of *Miscanthus* has not been reported yet. In this work, primary phenols production after combined TFA pretreatment was identified. Hydrolysates by combined MA pretreatment were not characterized since massive presence of MA significantly affected the quantitative characterization of phenolic compounds.

Figure 3.5 presented six major identified phenolic compounds with concentration greater than 0.01 g/L in the hydrolysates. Some other omnipresent phenols found in biomass treated hydrolysates in other reports like 4-hydroxybenzoic acid, coniferyl aldehyde, and syringic acid were also detected, but with only trace amount. Among major phenols products, two hydroxycinnamic acids (p-coumaric and ferulic acids) constituted the largest fractions, with concentration of p-coumaric acid generally at least three times higher than that of ferulic acid. In herbaceous plants like *Miscanthus*, these two hydroxycinnamic acids link lignin and polysaccharides to form a unique lignin/phenolics-carbohydrate complex through ester and ether bonds (Lewis and Yamamoto, 1990). In addition, lignin consists of three monolignols with p-hydroxyphenyl (H), guaiacyl (G), and syringyl(S) moieties, respectively. In the hydrolysates of *Miscanthus* here, the phenolic compounds but p-coumaric and ferulic acid came from all three monolignol units, with p-hydroxybenzaldehyde belonging to H, vanillin and vanillic acid to G, and syringaldehyde to S. Additionally, as can be observed from Figure 3.5, it was important to notice that concentrations of phenols did not change considerably in the hydrolysates after combined acid and single H_2SO_4 pretreatment. On the contrary, single TFA

pretreatment resulted in significant accumulation of major identified phenolic compounds, with yields increased by 100-190%. The results suggested that compared to H_2SO_4, biomimetic acids could easily break down lignin and lignin-carbohydrate complexes and release lignin-derived compounds. This function was possibly due to their carboxylic structures which would esterify carbohydrate in lignin-carbohydrate complexes. Therefore, low biomimetic acids percentage would be suggested during a combined acid pretreatment to keep a low level of phenols yield.

3.3.5 Fermentability of Combined Acids Catalyzed Hydrolysates

Ethanol production from hemicellulose is not only dependent on xylose yield, but also on the fermentability of the treated hydrolysates. Therefore, in this work, five representative hydrolysate samples after filtration were chosen for fermentability tests in order to investigate the potential compound inhibitory effects of pretreatment degradation products on fermentation. These five samples included three hydrolysates after three single acid pretreatments (H_2SO_4, TFA, MA) as well as two samples after combined acid hydrolysis (50:50 H_2SO_4–TFA and 75:25 H_2SO_4-MA). The samples after combined acid hydrolysis were chosen for study in terms of their high xylose yields and low organic acid usage.

Over the fermentation, glucose of only trivial amount was completely consumed within 10 h while xylose was gradually utilized over the fermentation with various amount left in the solution depend on the type of hydrolysates and if overliming was employed beforehand. The maximum ethanol concentration was reached after 24 h. To better compare the fermentation results among various hydrolysate samples, major fermentation parameters were list in Table 3.3. In the case without overliming, for single H_2SO_4 pretreatment, ethanol yield was 0.166 g/g xylose present, much lower than that of 0.31-0.33 g/g from single xylose fermentation (Ha et al., 2011). This indicated that the significant presence of various degradation products in the hydrolysates greatly inhibited yeast growth and ethanol fermentation (ethanol yield per xylose utilized reflects fermentation efficiency while ethanol yield per xylose present is also related to yeast growth). Compared with single H_2SO_4 pretreatment, both TFA and MA pretreatment led to the plummeting of ethanol yield per xylose present (PXP). It clearly showed that these

biomimetic acid pretreatments greatly induced either higher degradation product yield or even new degradation products, which substantially inhibited fermentation. In contrast, in the case with oeverliming, all fermentation parameters changed substantially. For single H_2SO_4 pretreatment, ethanol yield increased to 0.185 g/g xylose present by 11%. Interestingly to note, both TFA and MA pretreatment obtained considerable improvement in ethanol yield PXP by 150-210% and 310-370%, respectively. Ethanol yields PXP of TFA pretreatments were brought up to the level resulted from single H_2SO_4 pretreatment, whereas those of MA pretreatments were even higher. Meanwhile, ethanol yields per xylose utilized (PXU) were mostly reduced to some extent compared with the cases without overliming. Specifically, compared with single H_2SO_4 pretreatment, both single and combined TFA pretreatment obtained considerably increased ethanol yields PXU to the level resulted from single xylose fermentation, but ethanol yields PXP were about the same. It can be concluded that in these hydrolysates, the degradation products inhibiting yeast growth were mainly removed rather than those inhibiting ethanol fermentation. If taking into account xylose yield obtained in the upstream pretreatment, TFA pretreatment increased overall ethanol yield by 27-38% compared to single H_2SO_4 pretreatment. In contrast, the case for MA pretreatment was different. Compared to single H_2SO_4 pretreatment, both single and combined MA pretreatment led to higher ethanol yield PXP but not ethanol yield PXU. This might be due to the fact that the degradation products inhibited both ethanol fermentation and yeast growth, but concentration of the products inhibiting yeast growth was mainly reduced. With both increased ethanol yield PXP and xylose yield in two stages individually, MA pretreatment improved overall ethanol yield by 54% compared to single H_2SO_4 pretreatment, higher than that by TFA pretreatment.

The inhibitory effects detected in the acid-catalyzed hydrolysates were attributed to several types of degradation products (weak acids, furans and phenolic compounds) and probably even the applied organic acids themselves. In addition, in the TFA/MA leading hydrolysates, metal ions may be introduced into the hydrolysates from the vessel wall of pretreatment reactors due to the strong chelation effect of carboxylic acid (Lee et al., 2011), and they exerted certain level of inhibition on the fermentation yeast. It has been tested in this work that at the applied concentration during the pretreatment, both biomimetic acids did not exert inhibitory effects on ethanol yield. In the original

hydrolysates without overliming, metal ions may be the major inhibitors to remarkably bring down the ethanol yield PXP for TFA/MA pretreatment. Since all acid pretreatments resulted in similar furans production and acetate accumulation, these two types of degradation products should not account for the gaps of ethanol yield PXP between single H_2SO_4 and TFA/MA pretreatment. Besides, overliming did not alter phenols concentration while could effectively precipitate heavy metals such as iron (Ranatunga et al., 2000). Consequently, the considerable rising of ethanol yield PXP by TFA/MA pretreatment after overliming was possibly due to the efficient removal of metal ions dissolved in the hydrolysates, although further study is required for verification. In the post-overliming hydrolysates, since furans and acetate were all in similar level as stated previously, the variance in ethanol production and ethanol yield may only be largely attributed to phenols yields. In general, phenols severely inhibited yeast growth and ethanol production rate but not ethanol yields in *S. cerevisiae* (Klinke, Thomsen and Ahring, 2004). Therefore, the improvement of ethanol production by MA pretreatment might be due to the reduced production of phenolic compounds. In contrast, the reason of improved ethanol yield by TFA pretreatment needs further investigation. Furthermore, it was also important to note that the interaction effects among various degradation products including additive and antagonistically synergistic effects were not considered here, which would make the case more complicated.

Finally, when taking chemical cost into account, combined MA pretreatment easily stand out for its lower chemical cost and higher overall ethanol yield compared to single H_2SO_4 pretreatment and combined TFA pretreatment. Previously, application of biomimetic catalysts would increase the costs significantly, but here by using combined acid pretreatment, catalyst costs could be reduced considerably by 50% to 75%, while retaining a similar ethanol yield. Besides, it is important to point out that overliming is a necessary step for biomimetic acid pretreatment to make the whole process more cost effective, since only adding 1 cent/kg *Miscanthus* could increase the overall ethanol yield by 1.5-3.7 times.

3.4 Conclusions

Combined inorganic-biomimetic acids hydrolysis was proposed for hemicellulose

hydrolysis and shown to be an efficient method in terms of significantly improved ethanol yield and reduced catalyst costs. The combined acid catalysts would combine the cost advantage of sulfuric acid and hydrolysis selectivity advantage of biomimetic acids with inhibited xylose degradation. For combined TFA pretreatment, positive synergistic effects in hemicellulose decomposition further help increasing xylose yield and reducing phenols production. Through fermentation by engineered *S. cerevisiae*, TFA and MA pretreatments increase overall ethanol yield by 27-54% compared to H_2SO_4 pretreatment.

3.5 Acknowledgements

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3.7 Figures and Tables

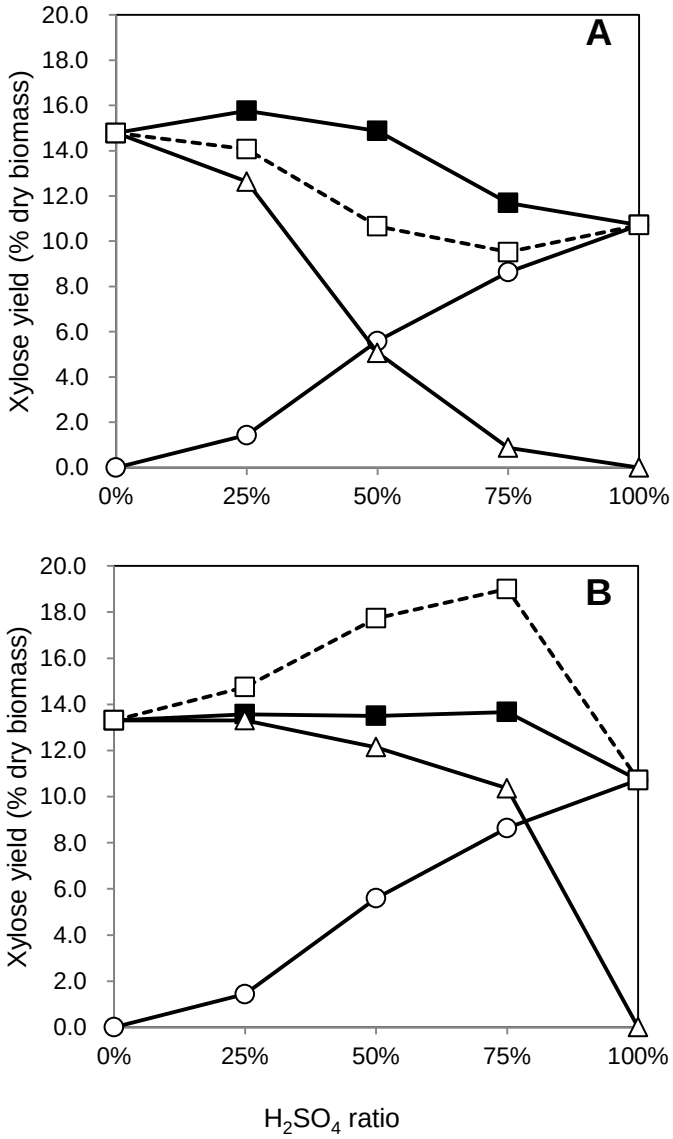

Figure 3.1. Xylose yield after pretreatment of *Miscanthus* by combined acid catalysts with varied acid blending ratios under optimal conditions (pH=1.07, 150 ºC, and 6.1 min). (A) H_2SO_4-TFA; (B) H_2SO_4-MA. (■) Compound effect of combined acid catalysts; (○) Individual effect of H_2SO_4 in the combined acid system; (△) Individual effect of TFA/MA in the combined acid system; (□) Hypothetical accumulative effects of individual effects between H_2SO_4 and TFA/MA.

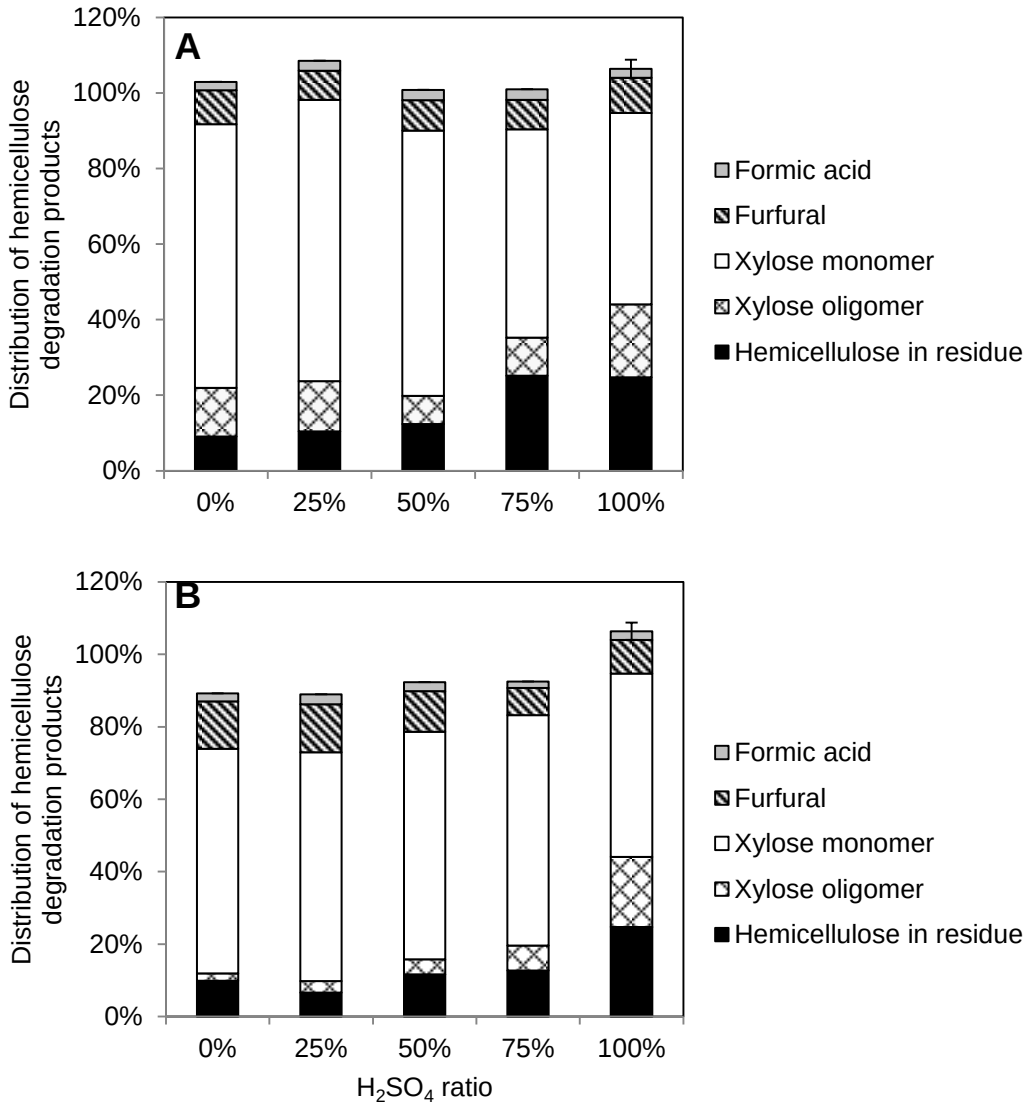

Figure 3.2. Distribution of hemicellulose degradation products after pretreatment under optimal conditions (pH=1.07, 150 ºC, and 6.1 min). (A) H_2SO_4-TFA; (B) H_2SO_4-MA. Recovery rate of hemicellulose related products ranges from 89.0% to 108.5%. Here the amount of all the degradation products was unified into xylose equivalent.

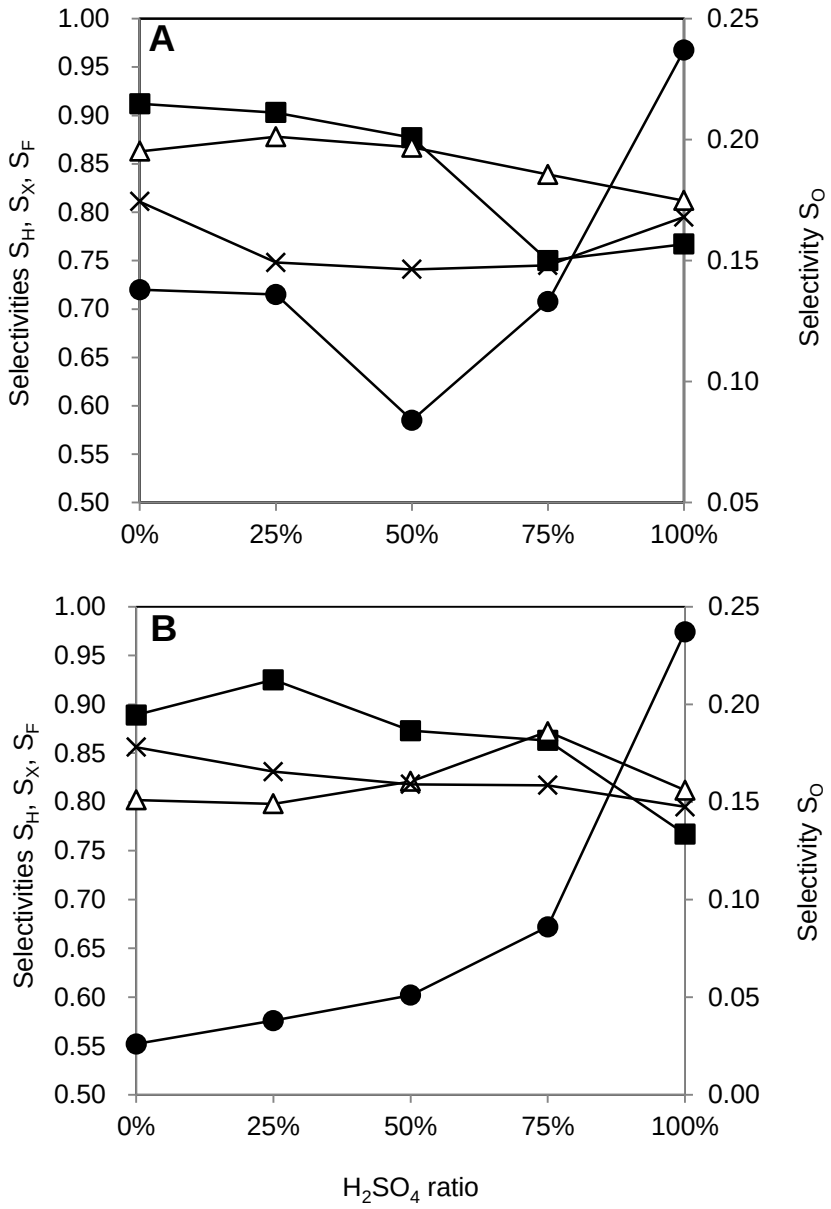

Figure 3.3. Selectivity of each reaction through hemicellulose hydrolysis. (A) H_2SO_4-TFA; (B) H_2SO_4-MA. S_H (■) refers to decomposition percentage of hemicellulose. S_X (△), S_F (×), and S_O (●) refer to reaction selectivities of monomeric xylose, furfural, and oligomeric xylose individually.

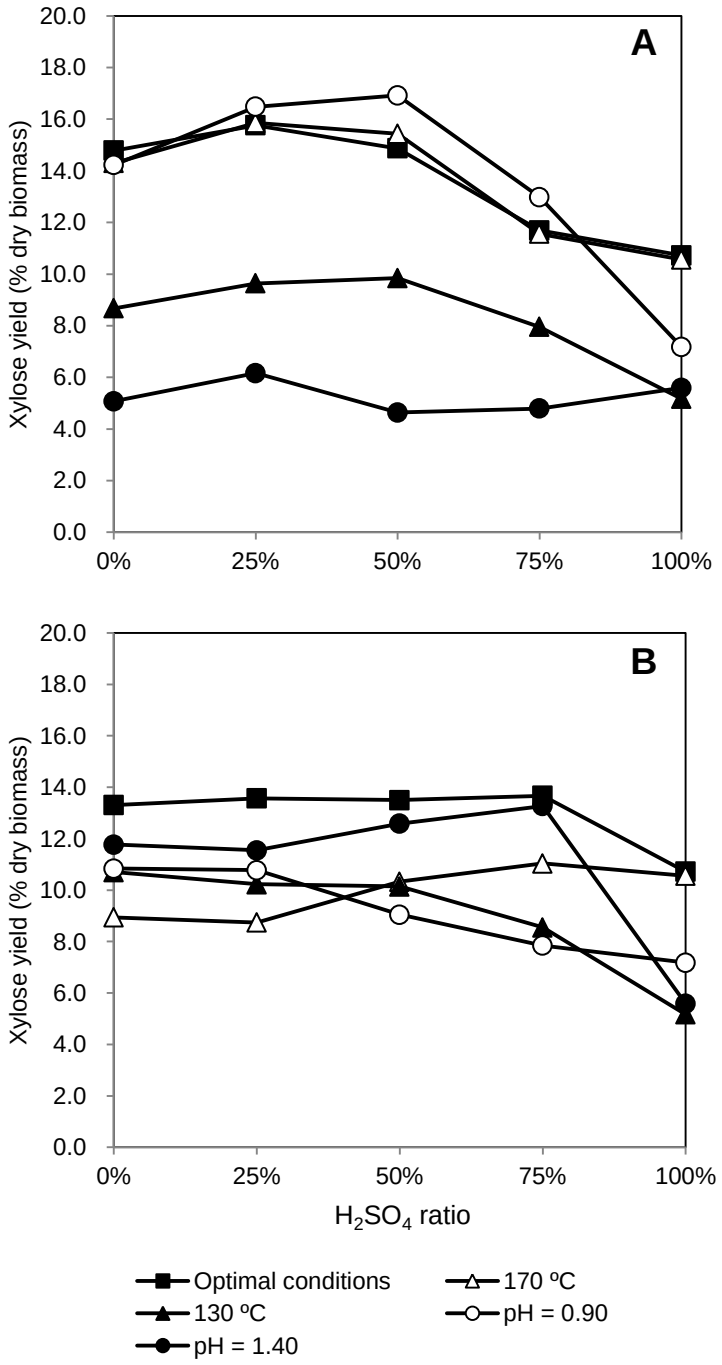

Figure 3.4. Xylose yield after pretreatment of *Miscanthus* by combined acid catalysts in function of temperature and acid dosage (Optimal conditions: pH=1.07, 150 ºC, and 6.1 min). (A) H_2SO_4-TFA; (B) H_2SO_4-MA.

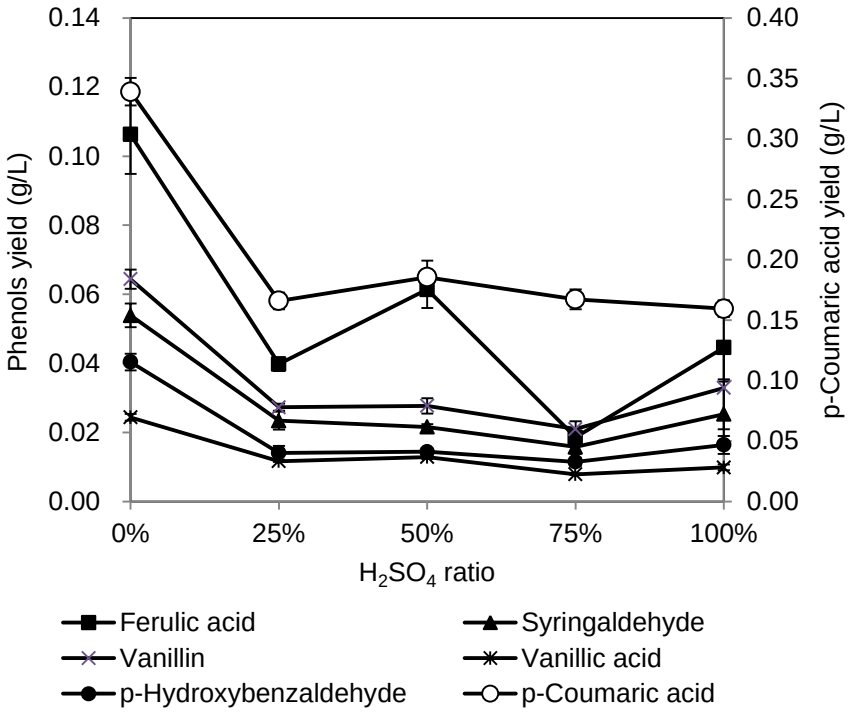

Figure 3.5. Phenolic compound production after combined TFA pretreatment under optimal conditions with various acid blending ratios.

Table 3.1. Furfural production by combined acid catalysis (in mg furfural/ml of hydrolysates)

Applied organic acid	H_2SO_4 ratio	Optimal conditions	170 ºC	130 ºC	pH 0.90	pH 1.40
TFA	0%	3.38	5.76	1.21	3.95	0.52
	25%	2.59	4.64	1.56	4.03	1.30
	50%	3.10	5.37	1.55	4.15	1.08
	75%	2.67	5.34	1.12	6.08	1.21
	100%	3.22	11.78	2.11	9.39	2.01
MA	0%	4.49	13.57	3.31	10.42	3.94
	25%	4.54	12.32	3.98	9.67	3.84
	50%	3.83	11.62	2.44	8.74	3.50
	75%	3.46	11.33	2.13	7.74	3.40
	100%	3.22	11.78	2.11	9.39	2.01

Table 3.2. Distribution of cellulose degradation products after pretreatment under optimal conditions (in % glucose equivalent).

Applied organic acid	H_2SO_4 ratio	Cellulose in residue	Glucose oligomer	Glucose monomer	HMF	Levulinic / formic acid	Recovery rate	Digestibility
TFA	0%	94.0	2.0	4.7	0.2	0.2	101.1	66.3
	25%	94.6	0.4	3.1	0.2	0.2	98.4	NA
	50%	97.1	NA	3.9	0.2	0.2	101.3	75.3
	75%	NA	1.8	4.7	0.2	0.1	NA	NA
	100%	93.9	2.4	3.9	0.2	0.5	100.9	69.7
MA	0%	90.2	0.4	5.4	0.5	0.4	97.1	53.6
	25%	92.8	0.6	5.5	0.4	0.4	99.8	69.5
	50%	87.9	0.8	4.9	0.4	0.4	94.3	69.7
	75%	97.4	0.6	3.1	0.1	0.3	101.6	NA
	100%	93.9	2.4	3.9	0.2	0.5	100.9	69.7

Table 3.3. Ethanol yield from various hydrolysates after fermentation by engineered *S. cerevisiae*. Standard deviations were within 5% range.

	Acid catalysts	Chemical cost (USD/Gal Ethanol *)	Ethanol yield (g/g xylose added)	Ethanol yield (g/g xylose utilized)	Xylose yield (g/g hemicellulose)	Overall ethanol yield (g/g hemicellulose)
No overliming	H_2SO_4	0.04	0.17	0.45	0.57	0.094
	50:50 H_2SO_4-TFA	2.29	0.07	0.34	0.75	0.054
	TFA	5.10	0.06	0.53	0.77	0.047
	75:25 H_2SO_4-MA	2.04	0.06	0.54	0.73	0.040
	MA	9.15	0.05	0.67	0.74	0.034
With overliming	H_2SO_4	0.10	0.19	0.29	0.57	0.105
	50:50 H_2SO_4-TFA	0.98	0.18	0.34	0.75	0.133
	TFA	1.70	0.19	0.35	0.77	0.145
	75:25 H_2SO_4-MA	0.54	0.22	0.30	0.73	0.162
	MA	1.97	0.22	0.28	0.74	0.161

* Chemical cost was expressed in US dollar per gallon of ethanol produced from the hemicellulose fraction in *Miscanthus*.

CHAPTER 4
CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

- A pretreatment method with successive acid and alkaline stages was developed, with the aim of co-utilization of cellulose and hemicellulose in the biomass.
- The best performance could be arrived at medium severities in the acid stage and high severities in the alkaline stage.
- In contrast to single stage pretreatments, acidic and alkaline pretreatments in succession could yield high recovery of both sugars (>80% glucose, >70% xylose), with significantly reduced formation of degradation by-products such as weak acids, furans and phenols.
- Combined inorganic-biomimetic acids hydrolysis was developed in the acid stage to improve hemicellulose hydrolysis.
- The combined acid catalysts would combine the cost advantage of sulfuric acid and hydrolysis selectivity advantage of biomimetic acids.
- Strong synergistic effects between the acid components in the combined system would facilitate decomposition of hemicellulose and oligomeric intermediates, and efficiently inhibit xylose degradation, and thereby increase xylose recovery.
- The combined catalysts would reduce catalyst costs and phenols production compared to biomimetic acid pretreatment.
- Two-stage acidic-alkaline pretreatment and combined acid hydrolysis would considerably increase overall ethanol yield, respectively.

4.2 Recommendations for Future Research

The current work has demonstrated the major achievement of the two pretreatment methods, but some detailed issues needed to be addressed to advance the technology development and pave the rode for future application. The primary problems were fleshed out as below.

1. **Techno-economic analysis of two-stage acidic-alkaline pretreatment**

As stated previously, pros and cons coexisted in the ACAL pretreatment method. A techno-economic analysis is desirable to compare the trade-offs and quantitatively evaluate the economic feasibility of the process. The work of Wingren et al. (2004) and Kadam et al. (2009) was great reference of the analysis on two-stage processes.

2. **Examine efficient pretreatment alternatives in each stage**

Lime pretreatment had its limitations in the two-stage pretreatment. Lime was less soluble in water at high temperatures, but NaOH avoided the problem. In addition, NaOH pretreatment would lead to more efficient delignification and decrystallization than lime pretreatment (Wang and Cheng, 2011). Therefore, NaOH could be employed to replace lime in the alkaline stage. Furthermore, alkali peroxide pretreatment could also be looked into, and it was expected to be effective even in mild conditions of reduced lime loading and probably reaction temperature (Yamashita et al., 2010).

3. **Kinetics study of combined acid hydrolysis**

Kinetic modeling analysis of hemicellulose decomposition and subsequent xylose degradation would be helpful to build an insightful understanding of the mechanism of combined acid hydrolysis, especially the unique function of individual acid component in the combined system. The effects of catalyst concentration, reaction temperature and time on the sequential process kinetics are recommended to investigate systematically. Besides, two acids could be added separately during the reaction to characterize their individual function. For instance, sulfuric acid is used solely at the beginning of reaction, and biomimetic acid is added halfway through the hydrolysis, and vise versa. Saeman models (consecutive pseudo-first-order models) and biphasic hydrolysis models (Lu and Mosier, 2008; Yat, Berger and Shonnard, 2008) could be used for the hydrolysis process simulation. The reaction rate constants and activation energy will be calculated and discussed further.

4. **Explore other options of biomimetic acid catalyst**

Extensive study is required to understand the effect of some other enzymatic structure and explore the possibilities for their application in design of biomimetic catalysts.

The key enzyme function domain contained two carboxylic acid residues. It was expected that the two acid residues was available right at the start of hydrolysis reaction

to ensure efficient general acid catalysis. Based on this observation, monocarboxylic acid and dicarboxylic acid should lead to different hydrolysis performance due to various numbers of present acid residues. Contrary to our expectation, both TFA (monocarboxylic acid) and MA (dicarboxylic acid) showed strong effects on the performance improvement. Therefore, other efficient and simple carboxylic acids should be tested to investigate the difference between two types of carboxylic acids. A good start would be acetic acid and malonic acid.

In addition, it has been noticed that the chemical mechanism throughout the enzymatic hydrolysis is highly correlated to the difference between the two acid residues. The hydrolysis process basically conducted two major mechanisms involving two types of enzyme portions: retaining enzyme and inverting enzyme. They had a short distance (4.8-5.3 Å) and long distance (9.0-10.5 Å), respectively (McCarter and Withers, 1994). Adequate distance between the two carboxylic groups should be positioned to provide enough room to house both water and substrate. Based on the structure characteristic, the functionality of biomimetic dicarboxylic acid during hydrolysis could also be affected by the length of carbon chain and the relative structural positions of two carboxylic groups. Therefore, dicarboxylic acids with different distances between the two carboxylic moieties and of different carbon chain could be also studied.

Furthermore, the tested dicarboxylic acids so far were all structural symmetric, so they contained two carboxylic groups with identical chemical features. However, in glycoside hydrolase, two critical carboxylic residues functioned differently as a proton donor and a nucleophile/base (McCarter and Withers, 1994). For that reason, in the biomimetic approach asymmetric dicarboxylic acids could be also applied to provide different functioning acid residues. Further, some cellulolytic enzymes had an essential Mg2+ in their active site (Jacobson et al., 1994), so the ion could be supplemented as part of the biomimetic catalysts by acting as a catalytic electrophile to facilitate proton transfer (Sinnott, 1990).

4.3 References

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APPENDIX A
RESPONSE SURFACE METHODOLOGY ANALYSIS

A1 RSM Results of Single Responses at Each Stage

The results of xylose yield after dilute sulfuric acid pretreatment in the acid stage in different tests were listed in Table A1. The regression equation of xylose yield was as follows:

$$y = 12.628 + 0.00125x_1 - 2.265x_2 - 1.1823x_3 - 1.2321x_1^2 - 1.7809x_2^2 - 0.4245x_3^2$$
$$- 1.575x_1x_2 - 0.525x_1x_3 - 1.135x_2x_3 + e \quad (R^2 = 0.97)$$

The quadratic model was tested for adequacy by the analysis of variance (ANOVA) and the results were shown in Table A2. The coefficient of determination, R^2 (1-SS_{err}/SS_{tot}), which measured the proportion of variance explained by the obtained results, was shown in the brackets following the equation. A high value of R^2 should be met and F_{model} should be at least four to five times the critical F ratio for the significance level 5% and (number of regression) and (number of residual) degrees of freedom (Box, Hunter and Hunter, 2005), to show the model is significant. Moreover, $F_{residual}$ ($MS_{lack\text{-}of\text{-}fit}$ / $MS_{pure\ error}$) should not exceed the critical F ratio for the significance level 5%, and (number of lack-of-fit) and (number of pure error) degrees of freedom, showing an insignificant lack of fit (Paiva, Maldonade and Scamparini, 2009).

As can be seen in Table A2, the model was highly significant since F_{model} (36.11) was much higher than $F_{9,10}$ (3.02). The coefficient of determination in the equation indicated 97% of the total variation could be explained by the model and it further testified the model adequately fit the obtained results. $F_{residual}$ (1.29) was lower than $F_{5,5}$ (5.05), did not show a significant lack of fit.

The results of total phenols after dilute sulfuric acid pretreatment in the acid stage in different tests were listed in Table A1. The regression equation of total phenols was as follows, and the analysis of variance of the regression model was shown in Table A3.

$$y = 3.08195 + 0.202x_1 + 0.4112x_2 + 0.0702x_3 + 0.0014x_1^2 + 0.0479x_2^2 + 0.0031x_3^2$$
$$- 0.1428x_1x_2 - 0.0243x_1x_3 - 0.0501x_2x_3 + e \quad (R^2 = 0.90)$$

The results of glucose yield after lime pretreatment in the alkaline stage in different tests were listed in Table A4. The regression equation of glucose yield was as follows, and the analysis of variance of the regression model was shown in Table A5.

$$y = 0.4498 - 0.00067x_1 - 0.04636x_2 - 0.02434x_3 - 0.15559x_1^2 + 0.087x_1x_2 + e$$

$$(R^2 = 0.96)$$

A2 Quantification of Inhibition Effects of Primary Degradation By-Products

Desirability function approach was applied in this work to optimize multiple responses of same interests simultaneously. In the approach, the value of each response y_i was scaled to a dimensionless value, d_i, called individual desirability. The scaling process of each response was interpreted employing the inhibition effect of correspondent degradation by-product. The desirability function was described as follows:

$$d = f(R)$$

Where d was the individual desirability of each by-product, as well as the percentage of the ethanol production rate when no inhibitory by-products was present; R was the response of each by-product, as well as the concentration of by-products in the fermentation hydrolysate; f was the function of inhibition effect of each by-product. The interactive effects of multiple by-products on sugar fermentation to ethanol were seldom studies. Here we assumed no combined inhibitory effects among the present inhibitory by-products. In addition, the inhibitory effects of by-products on xylose fermentation by *S. cerevisiae* were seldom investigated. Here we applied the inhibitory effects on glucose fermentation by *S. cerevisiae*, based on the assumption that the effects on both glucose and xylose fermentation by the yeast, or the engineered strain were the same.

The desirability functions of related inhibitory by-products were discussed individually as follow.

For furfural, various studies of inhibitory effects on ethanol production from xylose by *S. cerevisiae* were referred (Banerjee, Bhatnagar and Viswanathan, 1981; Boyer et al., 1992; Delgenes, Moletta and Navarro, 1996; Palmqvist, Almeida and Hahn-Hägerdal, 1999; Taherzadeh et al., 1999). Navarro (1994) found out the inhibitory effect was highly related to initial yeast concentration. With a high concentration of inoculums, the inhibitory effect caused by furfural was diminished, or even almost disappeared with

inoculum size higher than 9 g/L, based on the fact that furfural could be taken up and converted by yeast cells. In this study, we applied a medium level of inoculums concentration. Therefore, the fitted quadratic model of inhibition curve at 2-3 g/L inoculums size (Navarro, 1994) was used to describe the inhibitory effect of furfural as follows:

$$d_{Furfural} = 1 - (R_{Furfural}/7.5)^2$$

For HMF, two opposing factors were taken into account. On one hand, furfural had much stronger immediate inhibitory effect on yeast growth and fermentation than HMF (Sanchez and Bautista, 1988; Gao et al., 2006). HMF was allowed to have a concentration nearly twice as that of furfural, to achieve the same inhibitory effect induced by furfural. On the other hand, furfural and HMF could be converted by yeast cells to furfuryl alcohol, 5-hydroxymethylfulfuryl alcohol and 5-hydroxymethyl furan carboxylic acid (de Villegas, 1992), which were nontoxic to the yeast. In contrast to HMF, furfural was depleted much faster by the yeast, by a factor of approximately 4 in terms of the specific conversion rate (Taherzadeh et al., 2000). In this regard, HMF might exert severe problem than furfural for its extended effect throughout the fermentation. In this work, taking account of both factors, we employed the same desirability function as for furfural, with the only modification of 7.5 by dividing over 4/2:

$$d_{HMF} = 1 - (R_{HMF}/3.75)^2$$

For acetic acid, a number of studies (Pampulha and Loureiro, 1989; Phowchinda, Délia-Dupuy and Strehaiano, 1995; Taherzadeh, Niklasson and Lidén, 1997; Fernandes et al., 1997; Limtong et al., 2000) have been done on the inhibitory effects on ethanol production rate instead of ethanol yield from glucose by *S. cerevisiae*. Unlike furans, acetic acid was quite stable over the fermentation process, so it was assumed the inhibitory effect on ethanol production rate was as same as that on ethanol yield. Various quantifications of inhibitory effects by acetic acid were reported, and here we selected one typical interpretation (Phowchinda, Délia-Dupuy and Strehaiano, 1995) as follows:

$$P_i = P(1 - C_{Acetic\ Acid}/10)^{1.43}$$

Where P was ethanol production rate and C was concentration. In addition, xylose fermentation by recombinant *S. cerevisiae* was found to be much more sensitive to acetic

acid than glucose fermentation (Bellissimi et al., 2009). It was reported that at 2 g/L at pH 5, acetic acid led to a decline of ethanol yield from xylose by 50% (Helle et al., 2003). Here we kept the critical acetic acid concentration at 10 g/L, but changed the exponential power 1.43 to 3.1, to meet the requirement of 50% ethanol yield by 2 g/L acetic acid. Therefore, the desirability function of acetic acid was shown as follows:

$$d_{Acetic\_Acid} = (1 - R_{Acetic\ Acid}/10)^{3.1}$$

For formic and levulinic acids, the inhibitor effects on ethanol yield described previously were followed (Larsson et al., 1999). Their desirability functions were shown as follow:

$R = (0, 0.05)$ $\quad d = 2 \times R + 1$

$R = (0.05, 0.1)$ $\quad d = -2 \times R + 1.2$

$R > 0.1$ $\quad d_{Formic\ Acid} = -0.76 \times R_{Formic\ Acid} + 1.08$

$\quad d_{LevulinicAcid} = -0.46 \times R_{LevulinicAcid} + 1.05$

A3 RSM Results of Multiple Responses at Each Stage

The overall desirability of furans and acetate after dilute sulfuric acid pretreatment in the acid stage was calculated by the following equation:

$$D = (d_{Furfural} \times d_{HMF} \times d_{Acetic\ Acid})^{1/3}$$

The results under various conditions were listed in Table A6. The regression equation of overall desirability was as follows, and the analysis of variance of the regression model was shown in Table A7.

$$D = 0.03955 - 0.1194x_1 - 0.1237x_2 - 0.0587x_3 + 0.0643x_1^2 + 0.0415x_2^2 + 0.0211x_3^2 + 0.0687x_1x_2 + 0.0137x_1x_3 + 0.0223x_2x_3 + e \qquad (R^2 = 0.94)$$

The overall weak acids desirability after lime pretreatment in the alkaline stage was calculated by the following equation:

$$D = (d_{Formic\ Acid} \times d_{LevulinicAcid} \times d_{Acetic\ Acid})^{1/3}$$

The results under various conditions were listed in Table A8. The regression equation of overall weak acids desirability was as follows, and the analysis of variance of the regression model was shown in Table A9.

$$y = 0.983 - 0.02023x_1 - 0.00625x_2 + 0.0087x_1^2 + 0.00273x_2^2 - 0.00487x_1x_2$$

$$(R^2 = 0.85)$$

The overall furans desirability after lime pretreatment in the alkaline stage was calculated by the following equation:

$$D = (d_{Furfural} \times d_{HMF})^{1/2}$$

The results under various conditions were listed in Table A10. The regression equation of overall furans desirability was as follows, and the analysis of variance of the regression model was shown in Table A11.

$$y = 0.99512 + 0.12883x_1 - 0.04433x_2 - 0.11156x_1^2 + 0.00976x_2^2 + 0.08256x_1x_2$$

$$(R^2 = 0.89)$$

A4 Overall Optimization at Acid Stage

For the dilute acid pretreatment in the acid stage, xylose yield and production of primary by-products had different profiles under various conditions. Therefore, a compromise was required to be made between them for the process optimization. The optimization was simplified as to seek for the maximized value of the overall desirability of furans and acetate. Meanwhile, the exploited domain was confined by two boundary conditions:

(1) Xylose yield was required to be at least 95% of maximal yield (13.17% dry biomass);

(2) $x_1^2 + x_2^2 + x_3^2 \le 2^2$. Within this spherical domain, the regression model was precise enough to interpret the function.

In fact, we could change the boundary of xylose yield from 95% down to 90%, 85%, etc. The optimization problem was solved by Microsoft Excel 2007 Solver, and the optimal conditions by different requirements were shown in Table A12. It showed that if reducing xylose yield by only 10% to 12.47% dry biomass, the newly located optimal conditions would result in much less by-products formation: roughly 50% less furfural and 25% less acetic acid. However, further sacrifice of xylose yield would not lead to significant reduction of by-products accumulation. Therefore, the study selected the optimal conditions at (-0.086, -0.660, -1.886) when the maximal xylose yield was 90% of

maximal yield (12.47% dry biomass). The real values of the optimal conditions were 0.73 wt% sulfuric acid, 150 ºC, and 6.1 min.

A5 References

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A6 Tables

Table A1. Values of responses after dilute acid pretreatment under various conditions

Run	Variables			Responses				
	AD x_1	T x_2	RT x_3	Xylose yield (% dry biomass)	Furfural conc. (g/L)	HMF conc. (g/L)	Acetic acid conc. (g/L)	Total phenols (g/L in gallic acid)
1	1	1	1	2.37	12.37	1.916	15.32	3.75
2	1	1	-1	7.58	10.35	0.869	11.35	3.54
3	1	-1	1	12.06	4.91	0.205	8.81	3.36
4	1	-1	-1	13.47	3.00	0.126	7.99	3.17
5	-1	1	1	7.17	9.74	0.733	9.77	3.58
6	-1	1	-1	11.02	6.87	0.424	8.93	3.49
7	-1	-1	1	11.30	2.40	0.101	6.09	2.83
8	-1	-1	-1	9.87	1.54	0.077	4.72	2.33
9	2	0	0	8.51	9.48	0.534	11.59	3.38
10	-2	0	0	6.56	2.15	0.123	3.46	2.55
11	0	2	0	0.92	12.97	3.488	16.48	4.13
12	0	-2	0	9.76	1.45	0.039	5.39	2.18
13	0	0	2	9.63	8.61	0.381	9.90	3.01
14	0	0	-2	14.37	3.66	0.176	7.67	2.94
15	0	0	0	12.80	5.73	0.225	8.79	3.05
16	0	0	0	13.08	6.38	0.281	9.82	3.08
17	0	0	0	13.03	6.04	0.238	9.07	3.02
18	0	0	0	12.57	5.55	0.205	8.46	2.98
19	0	0	0	12.11	6.68	0.254	9.40	3.02
20	0	0	0	10.96	7.53	0.360	9.32	3.10

Table A2. Analysis of variance for the regression model of xylose yield

Source	Sum of squares	Degrees of freedom	Mean sum of squares	F_{model}	$F_{residue}$
Regression	238.0	9	26.45	36.11	
Residual	7.3	10	0.73		1.29
Lack-of-fit	4.1	5	0.82		
Pure error	3.2	5	0.64		
Total	245.4	19	--		

Table A3. Analysis of variance for the regression model of total phenols

Source	Sum of squares	Degrees of freedom	Mean sum of squares	F_{model}	$F_{residue}$
Regression	3.686	9	0.410	10.50	
Residual	0.390	10	0.039		19.20
Lack-of-fit	0.371	5	0.074		
Pure error	0.019	5	0.004		
Total	4.076	19	--		

Table A4. Values of responses after lime pretreatment under various conditions

Run	Variables		Responses					
	LC x_1	T x_2	Glucose yield (% dry biomass)	Furfural conc. (g/L)	HMF conc. (g/L)	Acetic acid conc. (g/L)	Formic acid conc. (g/L)	Levulinic acid conc. (g/L)
1	1	1	34.5	0.093	0.54	6.39	8.67	0.48
2	-1	-1	39.1	0.107	0.37	6.15	5.83	0.32
3	1	-1	24.4	0.013	0.06	6.96	9.23	0.25
4	-1	1	14.4	0.806	2.82	7.73	3.26	0.62
5	1.414	0	36.9	0.037	0.08	7.00	10.32	0.35
6	-1.414	0	41.1	3.053	3.11	4.85	1.61	0.45
7	0	1.414	4.8	0.348	0.24	8.86	4.99	0.89
8	0	-1.414	20.7	0.094	0.10	6.85	7.52	0.34
9	0	0	44.9	0.081	0.49	9.44	9.35	0.47
10	0	0	51.5	0.391	0.26	6.91	5.87	0.43
11	0	0	43.0	0.169	0.28	7.71	6.97	0.34
12	0	0	40.5	0.095	0.32	7.42	9.38	0.38
13	0	0	45.0	0.143	0.37	6.61	6.00	0.26

Table A5. Analysis of variance for the regression model of glucose yield

Source	Sum of squares	Degrees of freedom	Mean sum of squares	F_{model}	$F_{residue}$
Regression	0.216	5	0.0432	30.16	
Residual	0.010	7	0.0014		0.68
Lack-of-fit	0.003	3	0.0011		
Pure error	0.007	4	0.0017		
Total	0.226	12	--		

Table A6. Values of the overall desirability of furans and acetate after dilute acid pretreatment under different conditions

Run	Variables			Desirabilities			
	AD x_1	T x_2	RT x_3	d furfural	d HMF	d acetic acid	D
1	1	1	1	0.000	0.739	0.000	0.000
2	1	1	-1	0.000	0.946	0.000	0.000
3	1	-1	1	0.571	0.997	0.001	0.092
4	1	-1	-1	0.840	0.999	0.007	0.180
5	-1	1	1	0.000	0.962	0.000	0.000
6	-1	1	-1	0.161	0.987	0.001	0.054
7	-1	-1	1	0.898	0.999	0.054	0.365
8	-1	-1	-1	0.958	1.000	0.138	0.509
9	2	0	0	0.000	0.980	0.000	0.000
10	-2	0	0	0.918	0.999	0.268	0.626
11	0	2	0	0.000	0.135	0.000	0.000
12	0	-2	0	0.963	1.000	0.091	0.444
13	0	0	2	0.000	0.990	0.000	0.000
14	0	0	-2	0.762	0.998	0.011	0.203
15	0	0	0	0.416	0.996	0.001	0.084
16	0	0	0	0.276	0.994	0.000	0.010
17	0	0	0	0.351	0.996	0.001	0.061
18	0	0	0	0.452	0.997	0.003	0.111
19	0	0	0	0.207	0.995	0.000	0.032
20	0	0	0	0.000	0.991	0.000	0.000
21	0	0	2.5	0.000	0.969	0.000	0.000
22	0	0	-2.5	0.923	1.000	0.065	0.392

Table A7. Analysis of variance for the regression model of overall desirability of furans and acetate

Source	Sum of squares	Degrees of freedom	Mean sum of squares	F_{model}	$F_{residue}$
Regression	0.74	9	0.0823	19.76	
Residual	0.05	12	0.0042		2.85
Lack-of-fit	0.04	7	0.0057		
Pure error	0.01	5	0.0020		
Total	0.79	21	--		

Table A8. Values of overall weak acids desirability after lime pretreatment under different conditions

Run	Variables		Desirabilities			
	LC x_1	T x_2	d formic acid	d levulinic acid	d acetic acid	D
1	1	1	0.933	1.008	0.998	0.979
2	-1	-1	0.980	1.006	0.999	0.995
3	1	-1	0.923	1.004	0.995	0.974
4	-1	1	1.058	1.011	0.991	1.020
5	1.414	0	0.905	1.006	0.995	0.968
6	-1.414	0	1.070	1.008	1.038	1.038
7	0	1.414	0.994	1.015	0.986	0.998
8	0	-1.414	0.952	1.006	0.996	0.984
9	0	0	0.921	1.008	0.983	0.970
10	0	0	0.979	1.007	0.995	0.994
11	0	0	0.961	1.006	0.991	0.986
12	0	0	0.921	1.007	0.993	0.973
13	0	0	0.977	1.004	0.997	0.993

Table A9. Analysis of variance for the regression model of overall weak acids desirability

Source	Sum of squares	Degrees of freedom	Mean sum of squares	F_{model}	$F_{residue}$
Regression	0.00422	5	0.00085	7.91	
Residual	0.00075	7	0.00011		0.66
Lack-of-fit	0.00025	3	0.00008		
Pure error	0.00050	4	0.00013		
Total	0.00497	12	--		

Table A10. Values of overall furans desirability after lime pretreatment under different conditions

Run	Variables		Desirabilities		
	LC x_1	T x_2	d furfural	d HMF	D
1	1	1	1.000	0.979	0.990
2	-1	-1	1.000	0.990	0.995
3	1	-1	1.000	1.000	1.000
4	-1	1	0.989	0.433	0.654
5	1.414	0	1.000	1.000	1.000
6	-1.414	0	0.834	0.314	0.511
7	0	1.414	0.998	0.996	0.997
8	0	-1.414	1.000	0.999	1.000
9	0	0	1.000	0.983	0.991
10	0	0	0.998	0.995	0.996
11	0	0	1.000	0.994	0.997
12	0	0	1.000	0.993	0.996
13	0	0	1.000	0.990	0.995

Table A11. Analysis of variance for the regression model of overall furans desirability

Source	Sum of squares	Degrees of freedom	Mean sum of squares	F_{model}	$F_{residue}$
Regression	0.267	5	0.0533	11.44	
Residual	0.033	7	0.00466		2172
Lack-of-fit	0.033	3	0.010863		
Pure error	0.00002	4	0.000005		
Total	0.299	12	--		

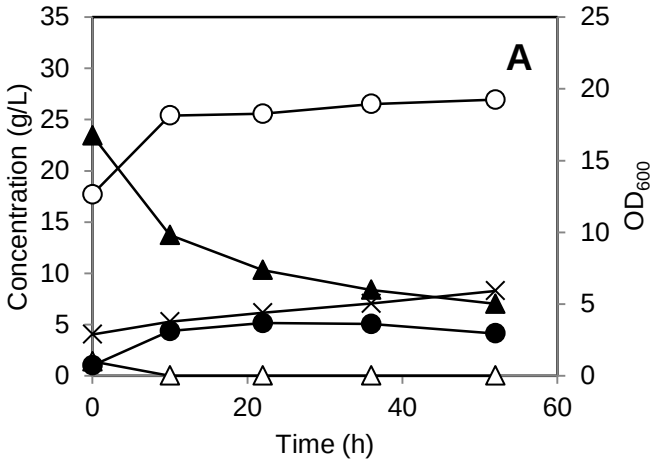
Table A12. Optimal conditions and the correspondent yields of xylose and degradation by-products

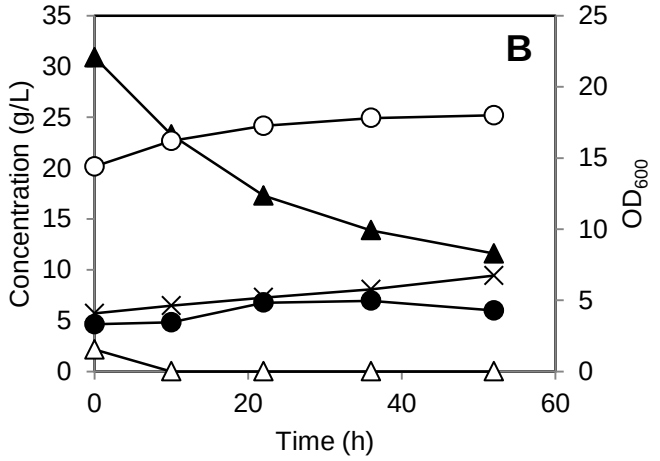
Optimal conditions	Xylose yield (% dry biomass)	Inhibitors production (mg/ml)		
		Furfural	HMF	Acetic acid
(0.580, -0.582, -0.973)	13.86	4.04	< 0.1	7.81
(0.219, -0.576, -1.903)	13.17 (95%)	2.53 (63%)	< 0.1	6.44 (82%)
(-0.086, -0.660, -1.886)	12.47 (90%)	1.95 (48%)	< 0.1	6.02 (77%)
(-0.318, -0.720, -1.839)	11.78 (85%)	1.55 (38%)	< 0.1	5.70 (73%)

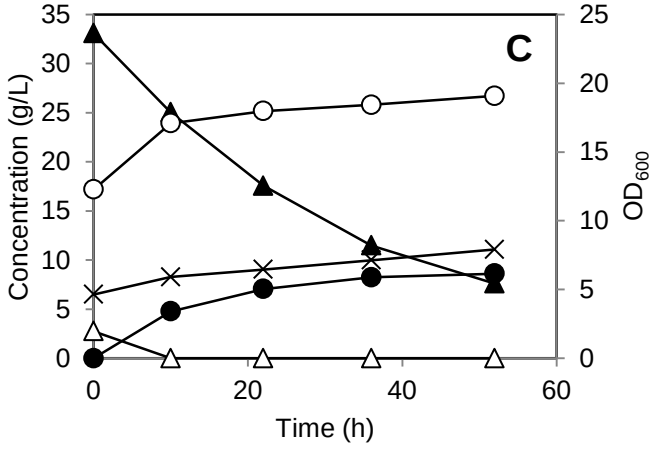
Note: the values in the bracket were the percentage of the values under the original optimal conditions at (0.580, -0.582, -0.973).

APPENDIX B

FERMENTATION RESULTS BY ENGINEERED *S. CEREVISIAE*







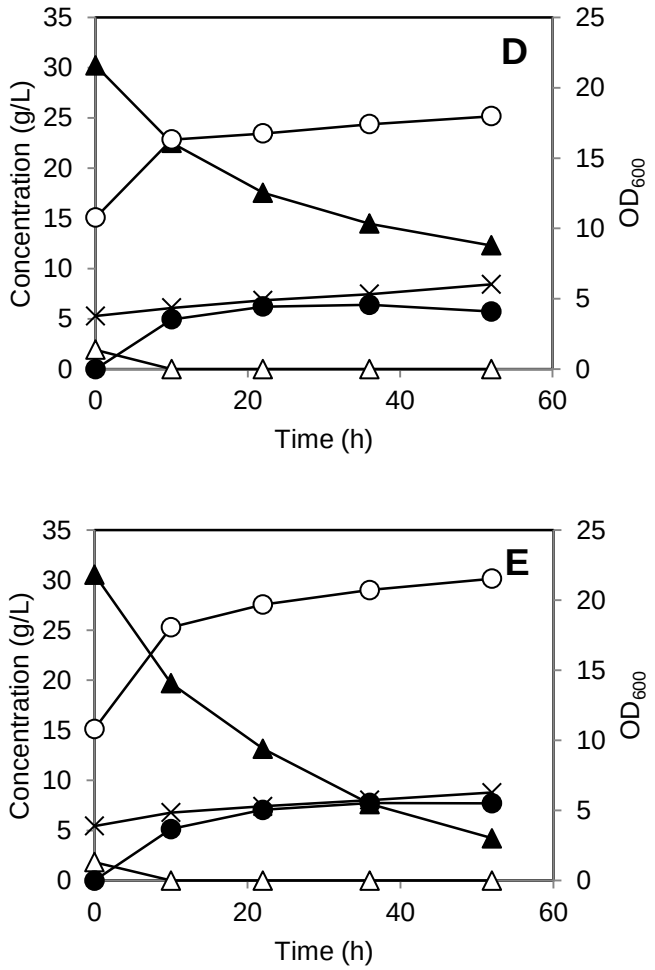


Figure B1. Fermentation profiles of combined acid-catalyzed hydrolysates by engineered *S. cerevisiae*. Standard deviations were within 5% range. (A) H_2SO_4; (B) TFA; (C) MA; (D) 50% H_2SO_4 - 50% TFA; (E) 75% H_2SO_4 - 25% MA. (○) OD_{600}; (▲) xylose; (△) glucose; (●) ethanol; (×) acetate.

Table B1. Effect of TFA and MA on the fermentation by engineered *S. cerevisiae* after 24 h. For all tests, initial OD_{600} = 5, [xylose] = 25 g/L, [glucose] = 2 g/L.

Added Acid	**Concentration of biomimetic acid (mol/L)**	**Specific growth rate of *S. cerevisiae* (h^{-1})**	**Ethanol yield (g/g sugars utilized)**
Control	NA	0.021	0.41
TFA	0.03	0.022	0.42
	0.05	0.024	0.48
	0.10	0.021	0.43
	0.21	0.019	0.43
MA	0.09	0.022	0.38
	0.19	0.021	0.46
	0.53	0.014	0.43
	1.21	0.006	NA*

* During fermentation with MA at 1.21 mol/L, nearly no xylose consumption was detected within 24 h, and ethanol was mainly produced from MA consumption.

APPENDIX C

MAJOR RECORDS OF ALL HYDROTHERMAL PRETREATMENT TESTS

Table C1. Major records of all hydrothermal pretreatment tests.

Test No.	Conduct Date	Reactor	Feedstocks	Catalysts	Operational Conditions
T00	Jul.03.2008	Model 4543	50g corn stover, 5% DM *	0.48 wt% H_2SO_4	160 ºC, 20 min, IP ** 95 psi
T01	Sep.17.2008	Model 4543	50g *Miscanthus*, 10% DM	0.48 wt% H_2SO_4	160 ºC, 20 min; then 240 ºC, 10 min. IP 95 psi
T02	Sep.22.2008	Model 4543	50g *Miscanthus*, 10% DM in the 1st stage, 20% DM in the 2nd stage	0.48 wt% H_2SO_4 in the 1st stage; 1:1 NH_3 loading in the 2nd stage	160 ºC, 20 min in the 1st stage; then 240 ºC, 10 min in the 2nd stage. IP 94 psi
T03	Sep.24.2008	Model 4543	50g *Miscanthus*, 28% DM	1:1 NH_3 loading	160 ºC, 20 min; then 240 ºC, 10 min. IP 95 psi
T04	Feb.13.2009	Model 4543	50g *Miscanthus*, 22% DM	1:1 NH_3 loading	240 ºC, 20 min, IP 96 psi
T05	Feb.13.2009	Model 4543	50g *Miscanthus*, 10% DM	0.48 wt% H_2SO_4	160 ºC, 20 min, IP 96 psi
T06	Feb.14.2009	Model 4543	100g *Miscanthus*, 22% DM	1:1 NH_3 loading	240 ºC, 10 min, IP 95 psi
T07	Feb.14.2009	Model 4543	50g *Miscanthus*, 10% DM	0.48 wt% H_2SO_4	160 ºC, 30 min, IP 95 psi
T08	May.23.2009	Model 4543	120g *Miscanthus*, 20% DM	1.0 wt% H_2SO_4	180 ºC, 35 min, IP 94 psi
T09	Jun.07.2009	Model 4543	120g *Miscanthus*, 20% DM	1.0 wt% H_2SO_4	180 ºC, 35 min, IP 94 psi
T10	Jun.08.2009	Model 4543	120g *Miscanthus*, 20% DM	1.0 wt% H_2SO_4	180 ºC, 35 min, IP 94 psi
T11	Jun.09.2009	Model 4543	120g *Miscanthus*, 20% DM	1.0 wt% H_2SO_4	160 ºC, 35 min, IP 94 psi
T12	Jun.10.2009	Model 4543	120g *Miscanthus*, 20% DM	1.0 wt% H_2SO_4	180 ºC, 35 min, IP 94 psi
T13	Jun.12.2009	Model 4543	120g *Miscanthus*, 20% DM	1.0 wt% H_2SO_4	180 ºC, 35 min, IP 95 psi
T14	Jun.13.2009	Model 4543	120g *Miscanthus*, 20% DM	1.0 wt% H_2SO_4	180 ºC, 20 min, IP 94 psi

Table C1. (Cont.)

Test No.	Conduct Date	Reactor	Feedstocks	Catalysts	Operational Conditions
T15	Jun.14.2009	Model 4543	120g *Miscanthus*, 20% DM	1.0 wt% H_2SO_4	170 ºC, 35 min, IP 94 psi
T16	Aug.08.2009	Model 4543	120g *Miscanthus*, 20% DM	0.75 wt% H_2SO_4	160 ºC, 25 min, IP 95 psi
T17	Aug.11.2009	Model 4543	120g *Miscanthus*, 20% DM	0.75 wt% H_2SO_4	160 ºC, 25 min, IP 94 psi
T18	Aug.13.2009	Model 4543	120g *Miscanthus*, 20% DM	0.75 wt% H_2SO_4	160 ºC, 25 min, IP 94 psi
T19	Aug.16.2009	Model 4543	120g *Miscanthus*, 20% DM	1.0 wt% H_2SO_4	175 ºC, 35 min, IP 94 psi
T20	Sep.07.2009	Model 4543	120g *Miscanthus*, 20% DM	1.0 wt% H_2SO_4	175 ºC, 15 min, IP 95 psi
T21	Sep.08.2009	Model 4543	120g *Miscanthus*, 20% DM	1.0 wt% H_2SO_4	145 ºC, 35 min, IP 95 psi
T22	Sep.11.2009	Model 4543	120g *Miscanthus*, 20% DM	1.0 wt% H_2SO_4	145 ºC, 15 min, IP 95 psi
T23	Sep.13.2009	Model 4543	120g *Miscanthus*, 20% DM	0.5 wt% H_2SO_4	175 ºC, 35 min, IP 94 psi
T24	Sep.16.2009	Model 4543	120g *Miscanthus*, 20% DM	0.5 wt% H_2SO_4	175 ºC, 15 min, IP 95 psi
T25	Sep.20.2009	Model 4543	120g *Miscanthus*, 20% DM	0.5 wt% H_2SO_4	145 ºC, 35 min, IP 94 psi
T26	Sep.22.2009	Model 4543	120g *Miscanthus*, 20% DM	0.5 wt% H_2SO_4	145 ºC, 15 min, IP 94 psi
T27	Sep.23.2009	Model 4543	120g *Miscanthus*, 20% DM	0.75 wt% H_2SO_4	130 ºC, 25 min, IP 94 psi
T28	Sep.24.2009	Model 4543	120g *Miscanthus*, 20% DM	0.75 wt% H_2SO_4	190 ºC, 25 min, IP 94 psi
T29	Sep.25.2009	Model 4543	120g *Miscanthus*, 20% DM	0.75 wt% H_2SO_4	160 ºC, 5 min, IP 94 psi
T30	Sep.27.2009	Model 4543	120g *Miscanthus*, 20% DM	0.75 wt% H_2SO_4	160 ºC, 45 min, IP 95 psi
T31	Sep.29.2009	Model 4543	120g *Miscanthus*, 20% DM	0.25 wt% H_2SO_4	160 ºC, 25 min, IP 95 psi
T32	Sep.30.2009	Model 4543	120g *Miscanthus*, 20% DM	1.25 wt% H_2SO_4	160 ºC, 25 min, IP 96 psi
T33	Oct.01.2009	Model 4543	120g *Miscanthus*, 20% DM	0.75 wt% H_2SO_4	130 ºC, 25 min, IP 94 psi

Table C1. (Cont.)

Test No.	Conduct Date	Reactor	Feedstocks	Catalysts	Operational Conditions
T34	Oct.20.2009	Model 4543	120g *Miscanthus*, 20% DM	0.75 wt% H_2SO_4	160 ºC, 25 min, IP 93 psi
T35	Oct.21.2009	Model 4543	120g *Miscanthus*, 20% DM	0.75 wt% H_2SO_4	160 ºC, 25 min, IP 88 psi
T36	Nov.15.2009	Model 4543	120g *Miscanthus*, 20% DM	0.75 wt% H_2SO_4	160 ºC, 0 min, IP 95 psi
T37	Nov.16.2009	Model 4543	120g *Miscanthus*, 20% DM	0.75 wt% H_2SO_4	160 ºC, 50 min, IP 95 psi
T38	Nov.17.2009	Model 4543	120g *Miscanthus*, 20% DM	0.75 wt% H_2SO_4	160 ºC, 0 min, IP 96 psi
T39	Dec.28.2009	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T40	Dec.29.2009	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T41	Dec.30.2009	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T42	Jan.01.2010	Model 4543	120g *Miscanthus*, 20% DM	8 ml/L TFA	150 ºC, 6 min, IP 95 psi
T43	Jan.02.2010	Model 4543	120g *Miscanthus*, 20% DM	7.5 ml/L MSA	150 ºC, 6 min, IP 95 psi
T44	Jan.03.2010	Model 4543	120g *Miscanthus*, 20% DM	4 ml/L TFA + 0.375 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T45	Jan.07.2010	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T46	May.27.2010	Model 4543	120g *Miscanthus*, 20% DM	2 ml/L TFA + 0.548 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T47	May.28.2010	Model 4543	120g *Miscanthus*, 20% DM	6 ml/L TFA + 0.183 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T48	May.30.2010	Model 4543	120g *Miscanthus*, 20% DM	2 ml/L TFA	150 ºC, 6 min, IP 96 psi
T49	May.31.2010	Model 4543	120g *Miscanthus*, 20% DM	4 ml/L TFA	150 ºC, 6 min, IP 96 psi
T50	Jun.03.2010	Model 4543	120g *Miscanthus*, 20% DM	6 ml/L TFA	150 ºC, 6 min, IP 95 psi
T51	Jun.03.2010	Model 4543	120g *Miscanthus*, 20% DM	NA	150 ºC, 6 min, IP 95 psi
T52	Jun.04.2010	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4 + 4.8 g cyclodextrin	150 ºC, 6 min, IP 95 psi

Table C1. (Cont.)

Test No.	Conduct Date	Reactor	Feedstocks	Catalysts	Operational Conditions
T53	Jun.05.2010	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4 + 0.96 g cyclodextrin	150 ºC, 6 min, IP 95 psi
T54	Jun.12.2010	Model 4543	120g *Miscanthus*, 20% DM	2 ml/L TFA + 0.548 wt% H_2SO_4	170 ºC, 6 min, IP 95 psi
T55	Jun.13.2010	Model 4543	120g *Miscanthus*, 20% DM	4 ml/L TFA + 0.365 wt% H_2SO_4	170 ºC, 6 min, IP 95 psi
T56	Jun.19.2010	Model 4543	120g *Miscanthus*, 20% DM	6 ml/L TFA + 0.183 wt% H_2SO_4	170 ºC, 6 min, IP 95 psi
T57	Jun.20.2010	Model 4543	120g *Miscanthus*, 20% DM	8 ml/L TFA	170 ºC, 6 min, IP 95 psi
T58	Jun.21.2010	Model 4543	120g *Miscanthus*, 20% DM	NA	170 ºC, 6 min, IP 95 psi
T59	Jun.22.2010	Model 4543	120g *Miscanthus*, 20% DM	2 ml/L TFA	170 ºC, 6 min, IP 95 psi
T60	Jun.23.2010	Model 4543	120g *Miscanthus*, 20% DM	4 ml/L TFA	170 ºC, 6 min, IP 95 psi
T61	Jun.24.2010	Model 4543	120g *Miscanthus*, 20% DM	6 ml/L TFA	170 ºC, 6 min, IP 95 psi
T62	Jun.26.2010	Model 4543	120g *Miscanthus*, 20% DM	2 ml/L TFA + 0.548 wt% H_2SO_4	130 ºC, 6 min, IP 95 psi
T63	Jul.08.2010	Model 4543	120g *Miscanthus*, 20% DM	4 ml/L TFA + 0.365 wt% H_2SO_4	130 ºC, 6 min, IP 94 psi
T64-2	Oct.19.2010	Model 4543	120g *Miscanthus*, 20% DM	6 ml/L TFA + 0.183 wt% H_2SO_4	130 ºC, 6 min, IP 96 psi
T65	Jul.09.2010	Model 4543	120g *Miscanthus*, 20% DM	8 ml/L TFA	130 ºC, 6 min, IP 94 psi
T66	Jul.12.2010	Model 4543	120g *Miscanthus*, 20% DM	6 ml/L TFA	130 ºC, 6 min, IP 94 psi
T67	Jul.13.2010	Model 4543	120g *Miscanthus*, 20% DM	4 ml/L TFA	130 ºC, 6 min, IP 95 psi
T68	Jul.13.2010	Model 4543	120g *Miscanthus*, 20% DM	2 ml/L TFA	130 ºC, 6 min, IP 95 psi
T69	Jul.14.2010	Model 4543	120g *Miscanthus*, 20% DM	1 ml/L TFA + 0.274 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T70	Jul.15.2010	Model 4543	120g *Miscanthus*, 20% DM	2 ml/L TFA + 0.183 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T71	Jul.16.2010	Model 4543	120g *Miscanthus*, 20% DM	3 ml/L TFA + 0.091 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi

Table C1. (Cont.)

Test No.	Conduct Date	Reactor	Feedstocks	Catalysts	Operational Conditions
T72	Jul.18.2010	Model 4543	120g *Miscanthus*, 20% DM	4 ml/L TFA + 1.095 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T73	Jul.19.2010	Model 4543	120g *Miscanthus*, 20% DM	8 ml/L TFA + 0.73 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T74	Jul.20.2010	Model 4543	120g *Miscanthus*, 20% DM	12 ml/L TFA + 0.365 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T75	Jul.21.2010	Model 4543	120g *Miscanthus*, 20% DM	16 ml/L TFA	150 ºC, 6 min, IP 95 psi
T76	Jul.22.2010	Model 4543	120g *Miscanthus*, 20% DM	12 ml/L TFA	150 ºC, 6 min, IP 95 psi
T77	Jul.17.2010	Model 4543	120g *Miscanthus*, 20% DM	3 ml/L TFA	150 ºC, 6 min, IP 95 psi
T78	Jul.23.2010	Model 4543	120g *Miscanthus*, 20% DM	1 ml/L TFA	150 ºC, 6 min, IP 95 psi
T79-2	Oct.07.2010	Model 4543	120g *Miscanthus*, 20% DM	15.4 g/L MA + 0.548 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T80-2	Oct.06.2010	Model 4543	120g *Miscanthus*, 20% DM	30.8 g/L MA + 0.365 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T81-2	Oct.07.2010	Model 4543	120g *Miscanthus*, 20% DM	46.1 g/L MA + 0.183 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T82-2	Oct.06.2010	Model 4543	120g *Miscanthus*, 20% DM	61.5 g/L MA	150 ºC, 6 min, IP 95 psi
T83-2	Oct.21.2010	Model 4543	120g *Miscanthus*, 20% DM	15.4 g/L MA	150 ºC, 6 min, IP 95 psi
T84-2	Oct.22.2010	Model 4543	120g *Miscanthus*, 20% DM	30.8 g/L MA	150 ºC, 6 min, IP 96 psi
T85-2	Oct.22.2010	Model 4543	120g *Miscanthus*, 20% DM	46.1 g/L MA	150 ºC, 6 min, IP 95 psi
T86-2	Oct.26.2010	Model 4543	120g *Miscanthus*, 20% DM	15.4 g/L MA + 0.548 wt% H_2SO_4	170 ºC, 6 min, IP 95 psi
T87-2	Oct.26.2010	Model 4543	120g *Miscanthus*, 20% DM	30.8 g/L MA + 0.365 wt% H_2SO_4	170 ºC, 6 min, IP 95 psi
T88-2	Nov.02.2010	Model 4543	120g *Miscanthus*, 20% DM	46.1 g/L MA + 0.183 wt% H_2SO_4	170 ºC, 6 min, IP 95 psi
T89-2	Nov.02.2010	Model 4543	120g *Miscanthus*, 20% DM	61.5 g/L MA	170 ºC, 6 min, IP 95 psi
T93	Oct.19.2010	Model 4543	120g *Miscanthus*, 20% DM	15.4 g/L MA + 0.548 wt% H_2SO_4	130 ºC, 6 min, IP 95 psi

Table C1. (Cont.)

Test No.	Conduct Date	Reactor	Feedstocks	Catalysts	Operational Conditions
T94	Oct.18.2010	Model 4543	120g *Miscanthus*, 20% DM	30.8 g/L MA + 0.365 wt% H_2SO_4	130 ºC, 6 min, IP 95 psi
T95	Oct.18.2010	Model 4543	120g *Miscanthus*, 20% DM	46.1 g/L MA + 0.183 wt% H_2SO_4	130 ºC, 6 min, IP 95 psi
T96	Oct.21.2010	Model 4543	120g *Miscanthus*, 20% DM	61.5 g/L MA	130 ºC, 6 min, IP 96 psi
T100	Nov.07.2010	Model 4543	120g *Miscanthus*, 20% DM	5.5 g/L MA + 0.274 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T101	Nov.07.2010	Model 4543	120g *Miscanthus*, 20% DM	11.0 g/L MA + 0.183 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T102	Nov.08.2010	Model 4543	120g *Miscanthus*, 20% DM	16.5 g/L MA + 0.091 wt% H_2SO_4	150 ºC, 6 min, IP 96 psi
T103	Nov.03.2010	Model 4543	120g *Miscanthus*, 20% DM	35.0 g/L MA + 1.095 wt% H_2SO_4	150 ºC, 6 min, IP 96 psi
T104	Nov.03.2010	Model 4543	120g *Miscanthus*, 20% DM	70.0 g/L MA + 0.73 wt% H_2SO_4	150 ºC, 6 min, IP 95 psi
T105	Nov.04.2010	Model 4543	120g *Miscanthus*, 20% DM	105.0 g/L MA + 0.365 wt% H_2SO_4	150 ºC, 6 min, IP 96 psi
T106	Nov.05.2010	Model 4543	120g *Miscanthus*, 20% DM	140.0 g/L MA	150 ºC, 6 min, IP 95 psi
T107	Nov.09.2010	Model 4543	120g *Miscanthus*, 20% DM	22.0 g/L MA	150 ºC, 6 min, IP 95 psi
T108	Dec.08.2010	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4	170 ºC, 6 min, IP 96 psi
T109	Dec.11.2010	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4	130 ºC, 6 min, IP 97 psi
T110	Dec.12.2010	Model 4543	120g *Miscanthus*, 20% DM	1.46 wt% H_2SO_4	150 ºC, 6 min, IP 97 psi
T111	Dec.13.2010	Model 4543	120g *Miscanthus*, 20% DM	0.365 wt% H_2SO_4	150 ºC, 6 min, IP 84 psi
T112	Jan.28.2011	Model 4543	120g *Miscanthus*, 20% DM	6 ml/L TFA + 0.183 wt% H_2SO_4	150 ºC, 6 min, IP 96 psi
T113	Apr.03.2011	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4	150 ºC, 6 min, IP 100 psi
T114	Apr.04.2011	Model 4543	120g *Miscanthus*, 20% DM	8 ml/L TFA	150 ºC, 6 min, IP 96 psi
T115	Apr.05.2011	Model 4543	120g *Miscanthus*, 20% DM	61.5 g/L MA	150 ºC, 6 min, IP 96 psi

Table C1. (Cont.)

Test No.	Conduct Date	Reactor	Feedstocks	Catalysts	Operational Conditions
T116	Apr.05.2011	Model 4543	120g *Miscanthus*, 20% DM	4 ml/L TFA + 0.365 wt% H_2SO_4	150 ºC, 6 min, IP 96 psi
T117	Apr.06.2011	Model 4543	120g *Miscanthus*, 20% DM	15.4 g/L MA + 0.548 wt% H_2SO_4	150 ºC, 6 min, IP 96 psi
T118	Apr.14.2011	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4	150 ºC, 6 min, IP 3 psi
T119	Apr.14.2011	Model 4543	120g *Miscanthus*, 20% DM	4 ml/L TFA + 0.365 wt% H_2SO_4	150 ºC, 6 min, IP 3 psi
T120	Jun.01.2011	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4	150 ºC, 6 min, IP 94 psi
T121	Jun.02.2011	Model 4543	120g *Miscanthus*, 20% DM	8 ml/L TFA	150 ºC, 6 min, IP 94 psi
T122	Aug.19.2011	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4	150 ºC, 6 min, IP 3 psi
T123	Aug.23.2011	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4	150 ºC, 6 min, IP 3 psi
T124 -A	Aug.30.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.091 g/g $Ca(OH)_2$/biomass	150 ºC, 30 min, IP 3 psi
T124 -B	Aug.30.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.091 g/g $Ca(OH)_2$/biomass	180 ºC, 30 min, IP 3 psi
T124 -C	Aug.30.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.091 g/g $Ca(OH)_2$/biomass	210 ºC, 30 min, IP 3 psi
T125	Aug.30.2011	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4	150 ºC, 6 min, IP 3 psi
T126	Aug.31.2011	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4	150 ºC, 6 min, IP 3 psi
T127	Aug.31.2011	Model 4543	120g *Miscanthus*, 20% DM	0.73 wt% H_2SO_4	150 ºC, 6 min, IP 3 psi
T128 -A	Oct.04.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.146 g/g $Ca(OH)_2$/biomass	180 ºC, 30 min, IP 3 psi
T128 -B	Oct.04.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.091 g/g $Ca(OH)_2$/biomass	250 ºC, 30 min, IP 3 psi
T128 -C	Oct.04.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.091 g/g $Ca(OH)_2$/biomass	230 ºC, 30 min, IP 3 psi
T129 -A	Oct.06.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.037 g/g $Ca(OH)_2$/biomass	210 ºC, 30 min, IP 3 psi
T129 -B	Oct.06.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.068 g/g $Ca(OH)_2$/biomass	210 ºC, 30 min, IP 3 psi

Table C1. (Cont.)

Test No.	Conduct Date	Reactor	Feedstocks	Catalysts	Operational Conditions
T129-C	Oct.06.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.119 g/g $Ca(OH)_2$/biomass	210 ºC, 30 min, IP 3 psi
T130-A	Oct.10.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.015 g/g $Ca(OH)_2$/biomass	210 ºC, 30 min, IP 3 psi
T130-B	Oct.10.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.015 g/g $Ca(OH)_2$/biomass	230 ºC, 30 min, IP 3 psi
T130-C	Oct.10.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.037 g/g $Ca(OH)_2$/biomass	230 ºC, 30 min, IP 3 psi
T131-A	Oct.12.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.037 g/g $Ca(OH)_2$/biomass	210 ºC, 20 min, IP 3 psi
T131-B	Oct.12.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.037 g/g $Ca(OH)_2$/biomass	210 ºC, 40 min, IP 3 psi
T131-C	Oct.12.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.037 g/g $Ca(OH)_2$/biomass	210 ºC, 50 min, IP 3 psi
T132-A	Oct.17.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.040 g/g $Ca(OH)_2$/biomass	210 ºC, 30 min, IP 3 psi
T132-B	Oct.17.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.068 g/g $Ca(OH)_2$/biomass	235 ºC, 30 min, IP 3 psi
T132-C	Oct.17.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.012 g/g $Ca(OH)_2$/biomass	235 ºC, 30 min, IP 3 psi
T133-A	Oct.18.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.040 g/g $Ca(OH)_2$/biomass	175 ºC, 30 min, IP 3 psi
T133-B	Oct.18.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.040 g/g $Ca(OH)_2$/biomass	210 ºC, 30 min, IP 3 psi
T133-C	Oct.18.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.012 g/g $Ca(OH)_2$/biomass	185 ºC, 30 min, IP 3 psi
T134-A	Oct.21.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.068 g/g $Ca(OH)_2$/biomass	185 ºC, 30 min, IP 3 psi
T134-B	Oct.21.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.080 g/g $Ca(OH)_2$/biomass	210 ºC, 30 min, IP 3 psi
T134-C	Oct.21.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.040 g/g $Ca(OH)_2$/biomass	210 ºC, 30 min, IP 3 psi
T135-A	Oct.22.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.068 g/g $Ca(OH)_2$/biomass	235 ºC, 30 min, IP 3 psi
T135-B	Oct.22.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.040 g/g $Ca(OH)_2$/biomass	245 ºC, 30 min, IP 3 psi
T135-C	Oct.22.2011	Model 4593	6.0g *Miscanthus*, 20% DM	NA	210 ºC, 30 min, IP 3 psi

Table C1. (Cont.)

Test No.	Conduct Date	Reactor	Feedstocks	Catalysts	Operational Conditions
T136-A	Oct.31.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.040 g/g $Ca(OH)_2$/biomass	210 ºC, 30 min, IP 3 psi
T136-B	Oct.31.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.012 g/g $Ca(OH)_2$/biomass	235 ºC, 30 min, IP 3 psi
T136-C	Oct.31.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.040 g/g $Ca(OH)_2$/biomass	245 ºC, 30 min, IP 3 psi
T137-A	Nov.01.2011	Model 4593	6.0g *Miscanthus*, 20% DM	NA	210 ºC, 30 min, IP 3 psi
T137-B	Nov.01.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.040 g/g $Ca(OH)_2$/biomass	210 ºC, 30 min, IP 3 psi
T137-C	Nov.01.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.080 g/g $Ca(OH)_2$/biomass	210 ºC, 30 min, IP 3 psi
T138-A	Nov.03.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.012 g/g $Ca(OH)_2$/biomass	185 ºC, 30 min, IP 3 psi
T138-B	Nov.03.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.068 g/g $Ca(OH)_2$/biomass	185 ºC, 30 min, IP 3 psi
T138-C	Nov.03.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.040 g/g $Ca(OH)_2$/biomass	175 ºC, 30 min, IP 3 psi
T139-A	Nov.17.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.003 g/g $Ca(OH)_2$/biomass	197 ºC, 30 min, IP 3 psi
T139-B	Nov.17.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.003 g/g $Ca(OH)_2$/biomass	197 ºC, 30 min, IP 3 psi
T139-C	Nov.17.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.003 g/g $Ca(OH)_2$/biomass	197 ºC, 10 min, IP 3 psi
T140-A	Nov.18.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.003 g/g $Ca(OH)_2$/biomass	197 ºC, 20 min, IP 3 psi
T140-B	Nov.18.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.003 g/g $Ca(OH)_2$/biomass	197 ºC, 40 min, IP 3 psi
T140-C	Nov.18.2011	Model 4593	6.0g *Miscanthus*, 20% DM	0.003 g/g $Ca(OH)_2$/biomass	197 ºC, 50 min, IP 3 psi
T141	Nov.23.2011	Model 4543	120g *Miscanthus*, 20% DM	4 ml/L TFA + 0.365 wt% H_2SO_4	150 ºC, 6 min, IP 3 psi
T142	Nov.27.2011	Model 4543	120g *Miscanthus*, 20% DM	15.4 g/L MA + 0.548 wt% H_2SO_4	150 ºC, 6 min, IP 3 psi
T143-A,B,C	Nov.27.2011	Model 4593	6.0g dry biomass from T122-T123-T125-T126-T127 residue mixture, 20% DM	0.024 g/g $Ca(OH)_2$/biomass	202 ºC, 30 min, IP 3 psi

Table C1. (Cont.)

Test No.	Conduct Date	Reactor	Feedstocks	Catalysts	Operational Conditions
T144	Nov.28.2011	Model 4543	120g *Miscanthus*, 20% DM	0.1 g/g $Ca(OH)_2$/biomass	120 ºC, 60 min, IP 3 psi
T145 - A,B, C	Nov.29.2011	Model 4593	6.0g dry biomass from T122-T123-T125-T126-T127 residue mixture, 20% DM	0.024 g/g $Ca(OH)_2$/biomass	202 ºC, 30 min, IP 3 psi
T146	Nov.29.2011	Model 4543	120g *Miscanthus*, 20% DM	0.024 g/g $Ca(OH)_2$/biomass	202 ºC, 30 min, IP 3 psi
T147 - A,B, C	Nov.29.2011	Model 4593	6.0g dry biomass from T141 residue, 20% DM	0.024 g/g $Ca(OH)_2$/biomass	202 ºC, 30 min, IP 3 psi
T148 - A,B, C	Nov.30.2011	Model 4593	6.0g dry biomass from T141 residue, 20% DM	0.024 g/g $Ca(OH)_2$/biomass	202 ºC, 30 min, IP 3 psi
T149	Dec.01.2011	Model 4543	120g *Miscanthus*, 20% DM	0.1 g/g $Ca(OH)_2$/biomass	120 ºC, 60 min, IP 3 psi
T150 - A,B, C	Dec.01.2011	Model 4593	6.0g dry biomass from T142 residue, 20% DM	0.024 g/g $Ca(OH)_2$/biomass	202 ºC, 30 min, IP 3 psi
T151	Dec.02.2011	Model 4543	120g *Miscanthus*, 20% DM	1.0 wt% H_2SO_4	180 ºC, 15 min, IP 3 psi
T152 - A,B, C	Dec.03.2011	Model 4593	6.0g dry biomass from T142 residue, 20% DM	0.024 g/g $Ca(OH)_2$/biomass	202 ºC, 30 min, IP 3 psi
T153	Dec.04.2011	Model 4543	120g *Miscanthus*, 20% DM	1.0 wt% H_2SO_4	170 ºC, 15 min, IP 3 psi
T154	Dec.10.2011	Model 4543	120g *Miscanthus*, 20% DM	8 ml/L TFA	150 ºC, 6 min, IP 3 psi
T155	Dec.11.2011	Model 4543	120g *Miscanthus*, 20% DM	61.5 g/L MA	150 ºC, 6 min, IP 3 psi

Note: * Dry matter content;

** Initial pressure when the reaction started.